

Research Article

Assessment of the shelf-life of *Nannochloropsis oculata* flocculates stored at different temperatures

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ABSTRACT. The cell and culture viability of concentrates of the microalga *Nannochloropsis oculata* were assessed after storage for a period of 16 weeks at -18, 0 and 5°C. The concentrates were obtained from the crop of *N. oculata*, which was harvested at the start of the seasonal growth period using a process of flocculation. Floccotac Plus was used as the flocculation agent, achieving flocculation of 90% of the suspended microalgae. It was observed that the chemical process did not affect the number of live cells. The concentrate stored at -18°C presented slow freezing, which deteriorated the cells and therefore reduced cell viability after five weeks (75%). The concentrates stored at 0 and 5°C showed cell viability over 97% after the 16 weeks. Culture viability was only seen in the concentrates stored at 0 and 5°C, which showed specific growth rates similar to those of the control culture. It may be concluded that it is possible to use flocculates stored at 0 and 5°C after 16 weeks as inoculum for mass crops of *N. oculata* for food, green water and other uses.

Keywords: *Nannochloropsis oculata*, shelf-life, flocculation, cell viability, aquaculture.

Evaluación de la vida útil de floculados de *Nannochloropsis oculata* almacenados a distintas temperaturas de conservación

RESUMEN. Se evaluó la viabilidad celular y de cultivo de concentrados de microalga *Nannochloropsis oculata*, almacenada durante 16 semanas a -18, 0 y 5°C. El concentrado se obtuvo de un cultivo de *N. oculata*, cosechado al inicio de la fase estacionaria de crecimiento, mediante un proceso de floculación. Como agente floculante se empleó Floccotac Plus, con el cual se logró una floculación del 90% de las microalgas suspendidas y el proceso químico no afectó el número de células vivas. El concentrado almacenado a -18°C experimentó un proceso de congelación lenta que deterioró las células y por ello redujo su viabilidad celular a cinco semanas (75%). Los concentrados almacenados a 0 y 5°C mostraron viabilidad celular superior al 97% al cabo de 16 semanas. Se observó viabilidad del cultivo sólo en los concentrados almacenados a 0 y 5°C, que presentaron tasas de crecimiento específico similares a las tasas del cultivo control. Se concluye que es posible utilizar, después de 16 semanas, floculados almacenados a 0 y 5°C como inóculo de cultivos masivos de *N. oculata* para alimento, aguas verdes y otros usos.

Palabras clave: *Nannochloropsis oculata*, vida útil, floculación, viabilidad celular, acuicultura.

INTRODUCTION

Cultivation of fresh microalgae is a limiting factor in the aquaculture industry in terms of the availability of foodstuffs. It is therefore recommended to evaluate supplements to fresh microalgae that are economically viable and which simplify the procedure of larva cultivation, such as the case of microalgae concentrates (D'Souza *et al.*, 2002). Supplements based on microalgae concentrated by flocculation or centrifugation can be a good alternative and appears to have the potential as

replacements for fresh food (Mc Causland *et al.*, 1999; Heasman *et al.*, 2000, 2001; Brown *et al.*, 2001; Knuckey *et al.*, 2006). Fresh microalgae can be substituted by up to 80% with concentrates from flocculation and stored for a maximum of 20 days (Brown *et al.*, 2001).

The process of cultivating microalgae requires special care against infection with pathogenic organisms or failure of the systems employed. If this occurs food may become scarce for the different aquaculture species kept in hatcheries. It is therefore

necessary to have specialized facilities and equipment in order to scale cultivations and thus avoid contamination and loss of microalgae. This equipment includes shelving installed in a special highly illuminated room for inoculation. Sterilization is carried out via physical methods (filtration, autoclave sterilization, pasteurization, UV radiation) or chemical methods (chloride, acidification, ozonation) (Jones *et al.*, 1993; Cysewski & Lorenz, 2006). Traditional microalgae cultivation requires specialized personnel, a large amount of space and other requirements that makes it more difficult (Table 1).

The microalga *Nannochloropsis oculata* is important in aquaculture due to its nutritional value. It belongs to the class Eustigmatophyceae, which groups together species that contain a high amount of polyunsaturated fatty acids (PUFAs), especially eicosa-pentaenoic acid (EPA), arachidonic acid (ARA) and docosahexaenoic acid (DHA), which are of great importance in the nutrition of marine animals, particularly for growth and development of the larvae of fish, mollusks and crustaceans (Otero *et al.*, 1997, Brown *et al.*, 1999). It is Japanese in origin and its size varies from 2 to 3 μm (Brown *et al.*, 1999). *N. oculata* has been suggested as a preferable foodstuff for the rotifer, *Brachionus plicatilis*, which efficiently transfers polyunsaturated fatty acids from the microalgae to marine fish larvae (Sukenik *et al.*, 1993, Hoshida *et al.*, 2005). In the study carried out by Brown *et al.* (1999), the cultures reached densities of 8×10^6 to 20×10^6 cell mL^{-1} and growth rates of 0.7 to 1.13 day^{-1} (López *et al.*, 2007; Sánchez-Torres *et al.*, 2008).

The imminent use of microalgae concentrates as a principal dietary component in the nutrition of aquatic organisms has been shown by several authors in juvenile *Pecten fumatus*, in larva and juvenile oysters *Crassostrea gigas* (Brown & Robert, 2002; Knuckey *et al.*, 2006). Heasman *et al.* (2001) and Brown *et al.* (2003) suggest that storing microalgae concentrates at low temperatures and in domestic refrigerators that are available in cultivation centers, may extend their shelf-life. As a result, the objective of this study was to evaluate the shelf-life of concentrates of *N. oculata* at different storage temperatures, in order to determine their subsequent use as foodstuff in the production of aquatic organisms and as inoculum in the cultivation of this type of microalgae.

MATERIALS AND METHODS

This research was carried out at the Experimental Aquaculture Laboratory, School of Marine Sciences, Pontificia Universidad Católica de Valparaíso, between January and June 2012. The microalgae *N. oculata* was

cultured in accordance with recommendations of Helm *et al.* (2006). The Guillard f/2 medium was used as fertilizer. The temperature used during the entire cultivation process was $18 \pm 2^\circ\text{C}$, with 24 h light (approximately 4000 lux) and aeration (Helm *et al.*, 2006). The culture was harvested at the start of the growth season when cell density was above 4.55×10^6 cell mL^{-1} .

Cell counting was performed daily using a microscope with a Neubauer hemocytometer. The specific growth rate (N° cell divisions/day) of the culture was used as the control rate to evaluate subsequent cultures (culture viability), applying the following equation (Odum & Barrett, 2006):

$$\text{TEC} = \frac{\ln N_t - \ln N_0}{t} \quad (1)$$

where, TEC = specific growth rate, $\ln N_t$ = natural log of the number of cells at time t (final), $\ln N_0$ = natural log of the number of cells at time $t = 0$ (inoculation) and t = time expressed in days of cultivation in each volume.

Flocculation

Flocculation was carried out with a modified version of the method by Brown *et al.* (2003) using Floccotac Plus[®] at 0,05%, a polyelectrolyte which chemical basis corresponds to an anionic polyacrylamide, as the flocculation agent. Once the culture had obtained the exponential phase, 20 L were transferred to a conical structure created specifically to facilitate the harvesting process (Fig. 1), then pH was adjusted to 10.2-10.4 with the addition of 2N NaOH and 40 ml of anionic polyelectrolyte were added. After 15 to 20 minutes, the microalgae flocs sedimented to the bottom of the container. The excess water was extracted from the upper part of the container. The pH of the concentrated cells was adjusted to 7.0 using 4N HCl.

The efficiency of the flocculation was evaluated using the following equation proposed by Zuharlida *et al.* (2009):

$$\text{Efficiency of flocculation/harvest (\%)} = \frac{C_i - C_f}{C_i} \times 100(2)$$

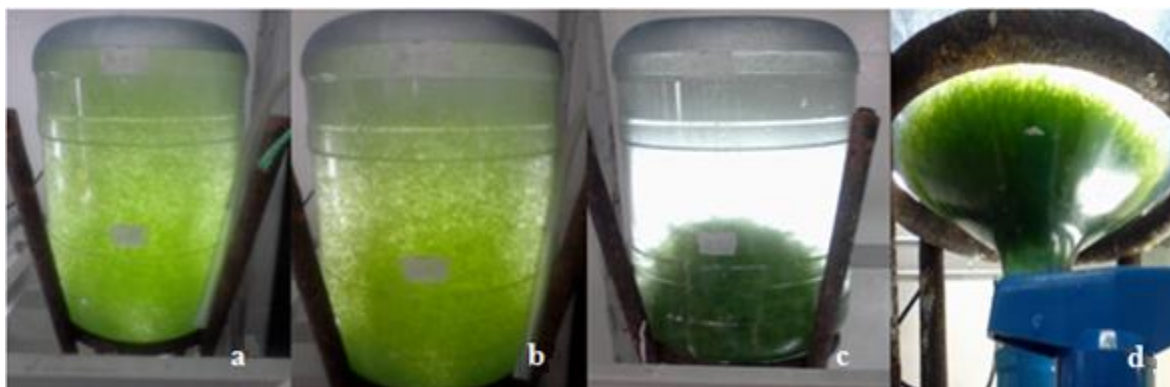
where, C_f = final concentration of cells in suspension and C_i = concentration of cells in suspension before the treatment.

Conservation of samples at different temperatures

The samples were stored at -18°C and refrigerated at 0 and 5°C (Brown *et al.*, 2003; Welladsen *et al.* (2014). For each temperature, 20 samples of 50 ml were stored without light or aeration for a maximum period of 16 weeks.

Table 1. Traditional microalgae cultivation *versus* concentrates.

Items	Flocculation concentrates	Traditional cultivation	Reference
Space availability	<	>	Heasman <i>et al.</i> (2001); Southgate & Lucas (2012)
Workforce	<	>	Heasman <i>et al.</i> (2001) ; Southgate & Lucas (2012)
Necessary materials and equipment	<	>	Jones <i>et al.</i> (1993); Cysewski & Lorenz (2006)
Risk of contamination	<	>	Jones <i>et al.</i> (1993); Cysewski & Lorenz (2006)
Growth of organisms (Bivalves)	<	>	Brown <i>et al.</i> (1999)

**Figure 1.** Addition and sedimentation of microalga cells through addition of a flocculant. a) Initiation of process, b) initiation of flocculation, c) flocs have grouped and sedimented to the center of the recipient, and d) sedimentation complete.

Cell viability

The cell viability of the flocculated microalga was evaluated via a staining protocol with Evans Blue Dye, as described by Heasman *et al.* (2001) and Zuharlida *et al.* (2009). This procedure consisted in extracting and homogenizing a sample of each of the flocculates stored at different temperatures; two cell counts were performed with a Neubauer hemocytometer. The samples were then stained with 0.5 mL of stock solution of Blue Evans Dye (1%) at 10 mL microalgae (Liu *et al.*, 2013). This was left for 15-20 min at room temperature, after which two further cell counts were performed at room temperature ($15 \pm 2^\circ\text{C}$) with a Neubauer hemocytometer, in order to observe the dead cells under a microscope. This procedure was conducted before and after flocculation and once a week on samples stored at each of the temperatures, until the end of the experiment. The percentage of viable cells was calculated using the equation proposed by Zuharlida *et al.* (2009) and Garzon-Sanabria *et al.* (2012):

$$\text{Cell viability (\%)} = \frac{N^\circ \text{ CV}}{N^\circ \text{ CT}} \times 100 \quad (3)$$

where, CT = total cells in the sample and CV = viable cells, those that are not affected by staining.

Culture viability

In order to evaluate culture viability, a sample of the stored flocculates ($-18, 0$ and 5°C) was extracted once a week. These samples were then used as an initial inoculum in a mass culture. The inoculum was added to a 2-L beaker of water, previously fertilized with Guillard f/2 medium, and maintained at $19 \pm 1^\circ\text{C}$ with 24 h aeration and light. The re-suspension of the flocculated microalgae was initially triggered by manual stirring; this was then followed by aeration stirring. This process was replicated in successive volumes of 5 and 20 L, in which a cell count was performed before and after replication using a Neubauer hemocytometer. The culture viability of the microalgae flocculates was evaluated using the specific growth rate (SGR).

Control culture

The mass culture of *N. oculata* used as control showed a specific growth rate for each culture volume as shown in Table 2. The mean growth rates obtained from the cultures with flocculation concentrates are compared with the SGR of the control culture. The final culture volume was 20 liters.

Table 2. Mean specific growth rate of the initial culture of *N. oculata*. SGR: specific rate expressed in N° of cell divisions/day.

Container/ volume (L)	Average SGR
Beaker (2)	0.593 ± 0.021
Bottle (5)	0.301 ± 0.014
Plastic box (20)	0.285 ± 0.032

RESULTS

Two 80 L culture bags of *N. oculata* were harvested with an approximate concentration of 4.55×10^6 cell mL⁻¹; these were labeled C1 = culture 1, and C2 = culture 2. The harvested volumes were reduced by more than 90% in comparison to their original volumes. A microscope was used to confirm that the cultures were harvested without any visible contamination. The efficiency of the flocculation process was: C1: 90.2% and C2: 91.9%. The staining protocol, both pre- and post-flocculation, did not show evidence that the cultures (C1 and C2) had damaged cells.

Variance analysis, with 95% confidence level, showed that there are significant differences when analyzing all temperatures jointly. The statistical result indicates that the group with the greatest difference is that which was stored at -18°C, while the flocculates stored at 0 and 5°C showed no significant differences between the viable cells of both groups.

The cells stored at -18°C showed cell viability up to 5 weeks, maintaining a level above 75% (Fig. 2). From week 6 to week 10, average cell viability for this temperature was close to 50%, and from week 12 to week 16, average cell viability was close to 10%. The samples stored at 0 and 5°C showed less than a 3% fall in total cell viability over time, obtaining a level over 97.5% cell viability for the entire period of study (16 weeks) (Fig. 2).

The cultures generated from flocculates stored at -18°C showed no culture viability after the first three weeks of study. Growth was seen during these first three weeks at growth rates of 0.23, 0.15 and 0.25, respectively, though this was only in the 2-L beakers. As a consequence of the above, the statistical analysis of the SGR was not carried out for the samples stored at -18°C. The cultures generated from flocculation concentrates of *N. oculata* stored at 0 and 5°C showed culture viability, with a specific growth rate fluctuating between 0.265 and 0.622 cell divisions/day, during the 16 weeks depending on the volume and the culture temperature (Table 3).

There is no significant difference, at a confidence level of 95%, between the growth rates of cultures from

Table 3. Mean specific growth rate (average of the 16 week) of cultures from concentrates stored at 0 and 5°C. SGR: specific rate expressed in N° of cell divisions/day.

Volume	Groups	Average SGR
Beaker 2 L	0°C	0.622 ± 0.016
	5°C	0.588 ± 0.015
Bottle 5 L	0°C	0.300 ± 0.005
	5°C	0.283 ± 0.003
Massive 20 L	0°C	0.265 ± 0.004
	5°C	0.280 ± 0.006

microalgae flocculates stored at 0 and 5°C, evaluating for the different culture volumes (2.5 and 20 L). With a confidence level of 95%, it can be seen that there is no significant difference between the mean specific growth rates of the initial culture and those generated from flocculates refrigerated at 0 and 5°C.

DISCUSSION

The initial culture of *N. oculata* was generated without any visible contamination, reaching 4.55×10^6 cell mL⁻¹ harvested at the start of the growth season. This cell concentration is below that achieved in other studies by Brown *et al.* (1999). The growth rate was close to 0.6 day⁻¹, which is slightly below that observed by López *et al.* (2007) and Sánchez-Torres *et al.* (2008), which was close to 0.7 day⁻¹. This difference may be due to the culture media used, the temperature, light intensity or CO₂ contribution, as these are generally different in each experiment.

The flocculation method used to concentrate the microalgae was considered successful, achieving a level of over 90% of the suspended cells from the initial culture. This is higher than the level reached by Knuckey *et al.* (2006), who concentrated 80% of cells with the same method, though using ferric chloride as the clarifying agent. This shows that Floccotac Plus is an appropriate flocculant for obtaining high flocculation percentages. This flocculant also allowed simple re-suspension with manual or mechanical stirring, which is difficult to achieve for the flocculation process as described by Knuckey *et al.* (2006). Samples are expected to be free of residual compounds of NaOH or HCl, given the dissociation generated to stabilize the pH at 7. It should be analyzed the amount of free acrylamide in the samples to evaluate if there is presence of this compound, however the amount of flocculant used in this experiment was very low (0.05%), so the residual is expected to be minimal as well.

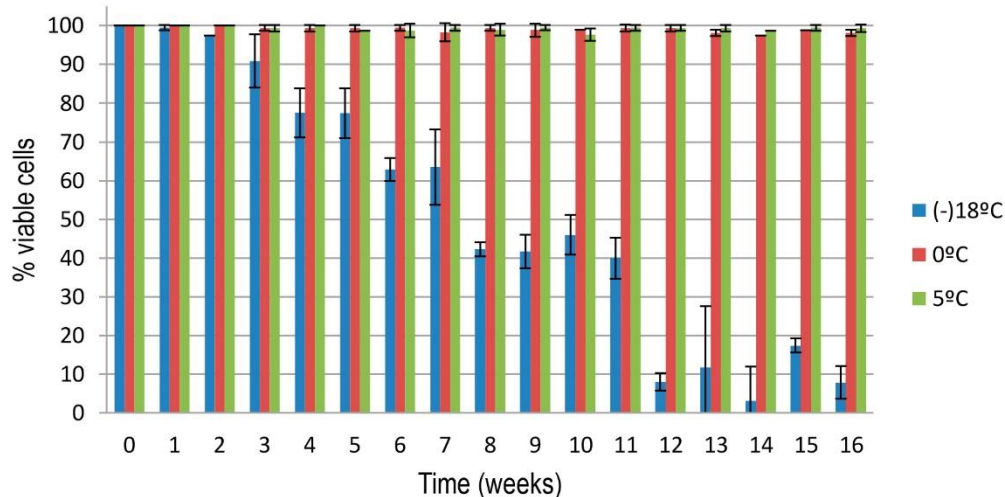


Figure 2. Percentage at which the concentrated cells maintain viability. Blue: samples stored at -18°C ; red: samples stored at 0°C ; green: samples stored at 5°C .

The flocculation concentrates were conserved using the storage methods of refrigeration and freezing, as suggested by Guerra *et al.* (2003) and Welladsen *et al.* (2014). This procedure did not lead to any contamination visible under a microscope. The temperatures used in this study were based on those suggested by Brown *et al.* (2003). Samples stored at -18°C showed a short shelf-life, this may be due to a process of slow freezing leading to weakness and subsequent rupture of the cell wall as the samples froze. This is shown by evaluation using a staining process over time.

Shelf-life was evaluated as cell viability and culture viability. For the samples stored at 0 and 5°C , both groups maintained cell viability of over 97% of total cells after 16 weeks. This result was higher than that obtained by Heasman *et al.* (2001) for several species of microalgae, where the maximum storage time was 15 weeks and cell viability reached 85%, using chitosan as the flocculation agent together with additives and preservatives. It was seen that the concentrate maintained its characteristics and could be used as food for an aquatic organism or to produce future cultures if necessary. This shows that the shelf-life as expressed by Bravo & Cueto (2001) and CENABAST (2011) is maintained.

The culture viability for both groups (0 and 5°C) was maintained throughout the 16 weeks, without significant differences ($P > 0.05$). It is therefore possible to maintain concentrates of *N. oculata* refrigerated in order to produce future cultures if necessary. This result provides unequivocal evidence of viability as stated by Castillo (2005), proving the continuity of shelf-life of 16 weeks or more.

The specific growth rates obtained for the cultures generated from the flocculation concentrates and refrigerated at 0 and 5°C showed no significant differences with the specific growth rates of the control culture, indicating that the SGR is not affected by the chemical process (flocculation) nor by the physical process (refrigeration) to which the cells are subjected, as the SGR results are similar to those obtained by López *et al.* (2007) and Sánchez-Torres *et al.* (2008).

D'Souza *et al.* (2002) recommend evaluating the length of the shelf-life of microalgae by concentration and refrigeration of the microalgae in order to simplify other cultures. McCausland *et al.* (1999), Heasman *et al.* (2000, 2001), Brown *et al.* (2001), Knuckey *et al.* (2006) mention replacing fresh food with microalgae concentrated by flocculation as a good alternative food source. Brown *et al.* (2001) has shown that microalgae concentrates can substitute fresh microalgae by up to 80%. Therefore, flocculation concentrates stored at low temperatures, such as 0 and 5°C , can extend their shelf-life to several weeks and are apt for use as food for different aquatic organisms. This study shows that this shelf-life can be extended to 16 weeks or more.

CONCLUSIONS

The anionic polyelectrolyte at 0.05% allows successful concentration of 90% of the cells of cultures of *N. oculata*. Flocculates stored at -18°C are viable for five weeks of storage, while flocculates stored at 0 and 5°C show cell viability and culture viability up to 16 weeks of conservation. After 16 weeks it is possible to use flocculates stored at 0 and 5°C as the inoculum for mass

cultures of *N. oculata* for food, green water and other uses in hatcheries of mollusks and fish larvae.

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