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Comparison of spore inoculated and vegetative propagated cultivation methods of *Gracilaria chilensis* in an integrated seaweed and fish cage culture

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Abstract. In Chile the integration of *Gracilaria chilensis* with salmon culture has shown high potential. Seaweed integrated aquaculture is of great interest as it allows waste recycling within fed cage aquaculture. The development of economically feasible suspended methods of seaweed cultivation is therefore of high importance. Hence, production and performance of two suspended *Gracilaria* cultivation methods, spore inoculated ropes and ropes with twined field collected seaweed, were studied in open water. The production from spore-seeded ropes was comparable to that of twined ropes for the first month of culture. Thereafter, the twined ropes had a significantly higher productivity. Fish farm wastes had no significant fertilizing effect upon *Gracilaria* growth rate. In addition, spore-originated thalli and field collected thalli were compared under laboratory conditions and in suspended culture using the same cultivation method. Spore-originated thalli had a 50% lower growth rate than the field collected thalli under laboratory conditions; however, no significant differences were detected in the field. Also, the occurrence of spore coalescence growth enhancement was not significant on the spore-seeded ropes. It was concluded that spore-originated cultivation techniques could be of interest for an integrated open seawater aquaculture system due to the high levels of *Gracilaria* polymorphism. This would result in greater adaptability to environmental variations, and a continuous supply of restocking material.

Introduction

Integrated seaweed/salmon cultivation has been suggested as a means of increasing overall production and decreasing nutrient waste in mariculture activities (Folke and Kautsky 1992; Troell et al. 1997, 1999; Naylor et al. 2000), and several studies have shown the potential of such integration particularly in land-based systems (see reviews in Troell et al. 2003; Neori et al. 2004). In Chile, the red seaweed *Gracilaria chilensis*, (Gracilariales, Rhodophyta; Bird et al. 1986), has been shown to be suitable for integration with salmon in both land-based tanks and in suspended cages (Buschmann et al. 1994; 1996b; Troell et al. 1997; Chow et al. 2001). *Gracilaria* growth in such systems more than doubled compared to monoculture due to fertilization effects (Buschmann et al. 2001). However, suspended culture methods that are effective and easy to manage need to be developed in order to make integrated aquaculture commercially viable. In Chile such methods are important as re-circulation and reduction of wastes may become a prerequisite to the further expansion of the salmon industry (Chopin et al. 2001).

Gracilaria chilensis is a major source for world agar production. Chile dominates global production with a yearly production of 70,000 wet tonnes, with 90% being cultivated as sterile vegetative thalli (i.e. clonal propagation) on intertidal or subtidal soft bottoms (Buschmann et al. 1995, 2001). Future expansion of *Gracilaria* bottom cultivation might be restricted by limited suitable cultivation areas, due to other space competing and the expanding of aquaculture operations, such as salmon and mollusc farming. (Buschmann et al. 1996a, 2001). Another restriction for *Gracilaria* bottom cultivation is the decrease in biomass production that usually occurs 2–3 years after establishment, presumably caused by repeated removal of the apical meristems. Epiphytes, mussel fouling, grazing and sedimentation can also impact negatively on production (Buschmann et al. 2001). Thus, there is a need to develop productive systems that do not loose their production potential over time.

Most studies of suspended *Gracilaria* cultivation in Chile have bundles of vegetative thalli tied to cultivation ropes (Pizarro and Barrales 1986; Westermeier et al. 1993). This practice is labour- and time-consuming, and inefficient, as large parts of the rope are left unproductive. Two different suspended methods which extend the cultivation efficiency along the whole rope and facilitate stocking procedure have been developed: One method uses thalli twined with three strings (Dr. Westermeier pers. com.), and an alternative technique uses spore-seeded ropes (Alveal et al. 1997), which may avoid the aging effect when replacing or replenishing old stands. With spore-seeding only a small amount of reproductive *Gracilaria* is needed to produce a large quantity of seeded ropes. The spore-seeding technique may also be of interest as coalescence plays a major role for *Gracilaria chilensis* sporeling survival (Santelices et al. 1999; Santelices 2001). Coalescence is a process that occurs in clonal seaweeds (species that can be propagated directly through regrowth of thalli fragments), where two or more sporelings fuse and develop an aggregate, within a common thickened wall. In the aggregate, the different sporelings form a bundle of thalli with genetically diversified axes. Under laboratory conditions polysporic, coalescent thalli have higher survival and higher production rates than non-coalescent thalli (Santelices et al. 1996, 1999). The coalescent sporelings are also expected to have increased resistance against grazers and infections (Santelices et al. 1999).

In Chile, the growing salmon industry may provide a large number of potential sites suitable for highly productive integrated farms in open waters. The main objective of this study was to compare different methods for cultivating *Gracilaria* on ropes, (1) grown-out spore-seeded ropes vs. ropes stocked with twined vegetative thalli, and (2) twisted ropes stocked with thalli emanating from spore-seeded ropes vs. twisted ropes stocked with vegetative thalli. Additionally, growth rates of coalescent, non-coalescent and vegetative thalli were compared and the effects of fish cage farm waste upon *Gracilaria* growth were observed.

Material and methods

The study was conducted in Metri Bay, situated 30 km south of Puerto Montt in southern Chile (Figure 1). Experimental work was carried out from February 2000–January 2001. Floating long-lines were placed at 30 m distance, on either side of a 150–200 t yr^{-1} mixed fish farm of coho salmon (*Oncorhynchus kisutch* Walbaum) and rainbow trout (*O. mykiss* Walbaum) and at a control site 300 m south of the farm (Figure 2). These distances were selected based on earlier results that demonstrated that *Gracilaria* could use the dissolved nitrogen in the water column to increase their growth and tissue nitrogen significantly (Troell et al. 1997). Cultivation ropes stocked with *Gracilaria* were

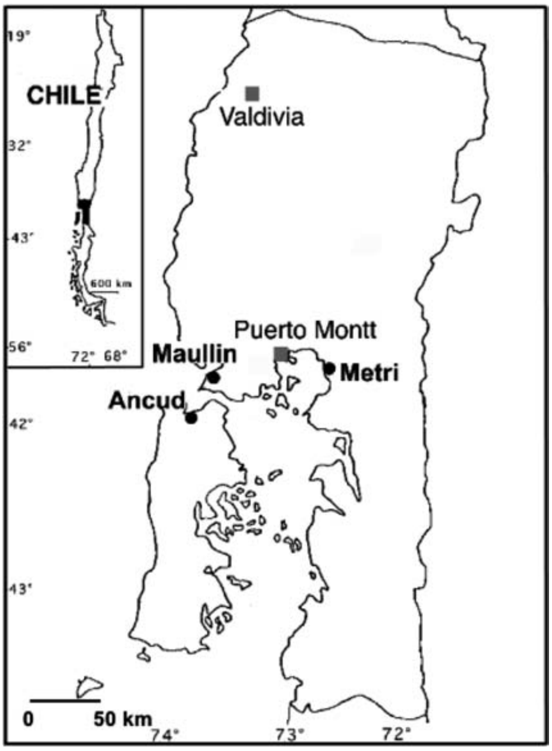

Figure 1. Map of the study area in Southern Chile showing the locations where the cultivated *Gracilaria* originated.

horizontally tied to metal frames (2.5 × 1.3 m) attached to long-lines. Each frame contained four ropes and the cultivation depth was kept constant at 1 m below the surface (Figure 3). Water depth below the ropes was 15–30 m with a maximum tidal amplitude of 7 m.

Specific growth rate (SGR % wet weight day^{-1}) was calculated after subtracting epiphytic load using the following formula:

$$\text{Specific Growth Rate(SGR)} = 100[\ln N_1/N_0)]/t_1$$

Where N_0 is the initial weight, N_1 the final weight and t is the cultivation time in days.

Experiment 1: Comparison of two suspended cultivation methods

Two different methods for rope cultivation were compared using (a) thalli originating from *Gracilaria* spores cultivated directly on inoculated ropes

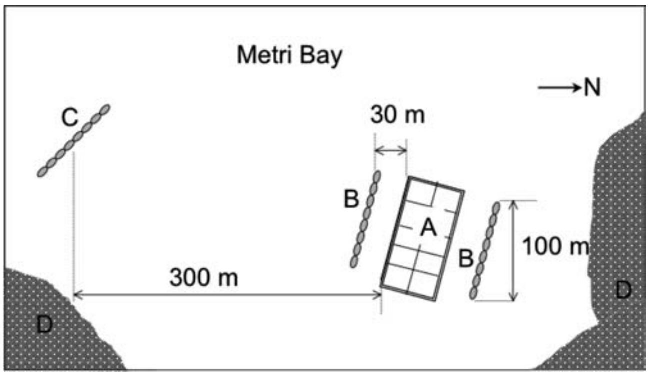

Figure 2. Schematic map showing the locations of the fish cages and the set-up of three long-lines in Metri Bay: (A) is the salmon cages; (B) are the two long-lines at 30 m distance; (C) is the long-line at 300 m distance; (D) is land bedrock

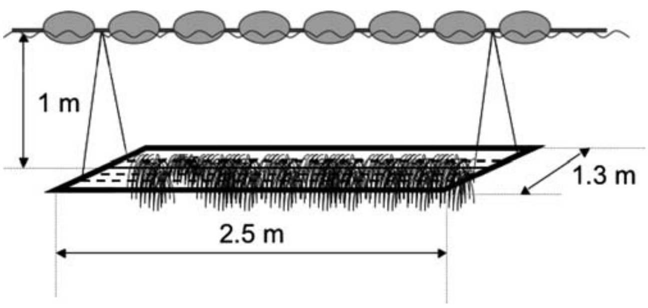

Figure 3. Seaweed frame, attached to the long-lines, containing four seaweed ropes.

(spore-seeded) and (b) cultivation ropes made of sterile field-collected thalli twined into ropes with three nylon strings (twined) (Figure 4). The study started in February 2000 and was finalised in October 2000. The spore-inoculated ropes were seeded in Ancud (situated on northern Chiloe Island) by using fertile material (tetrasporophytes or thalli with carposporophytes) collected from the Maullín area, 40 km west of Puerto Montt (Figuer 1). Thalli for the twined ropes were also collected from this area. Seven frames for each rope type were stocked with individually marked ropes and attached to the long-lines close to the fish cages and seven frames for each rope type were attached to the longline at the control site. The initial biomass was approximately 0.4 kg m^{-1} (1.0 kg m^{-2}) for the spore-seeded ropes and 0.2 kg m^{-1} (0.6 kg m^{-2}) for the twined ropes. Only spore-seeded ropes with homogeneous coverage with full-grown *Gracilaria* thalli (10–20 cm long) were used in the experiment. Five frames of each type at the two sites were harvested once a month. The other two frames were harvested bi-monthly in order to evaluate the impact of harvest frequency. Thalli from eight randomly selected samples of 0.5 m rope were weighed and removed from each treatment and site at every harvest and kept frozen for analyses of the epiphytic load. Only new production was harvested and around 15 cm of the thalli (approximately corresponding to the initial stocking by length) was left on the ropes for re-growth.

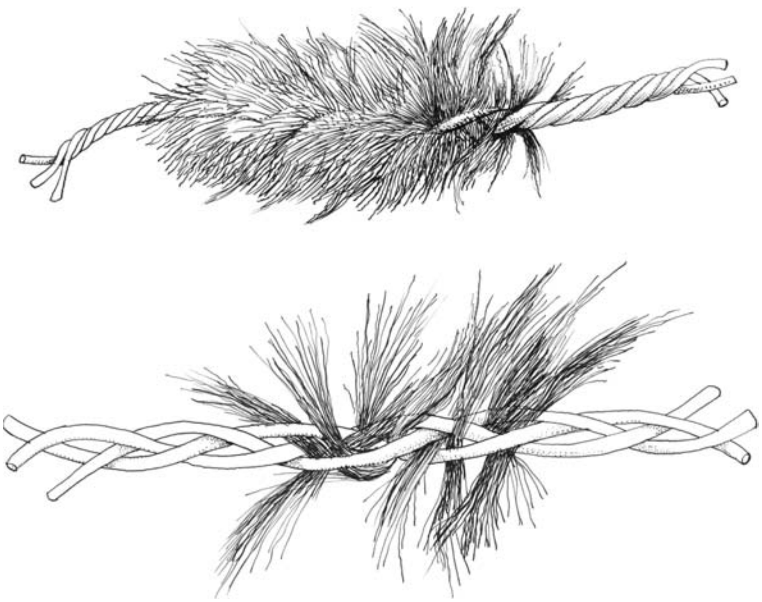

Figure 4. Schematic figure of the twined method (above) used in the first experiment for the comparison with spore-inoculated ropes; and the modified twisted method (below) used in the second experiment for cultivating and comparison of two thalli types.

Experiment 2: Two types of thalli tested using the twisted-rope cultivation method

Gracilaria from spore-seeded ropes (i.e. fresh thalli of known age) and collected thalli were harvested and twisted into ropes, using a modification of the twined i.e. inserting the thalli between the strands of the braid instead of being twined by winding (Figure 4). These ropes were attached to eight frames placed on long-lines close to the fish farm and at the control site. The initial weight for each rope was 0.8–1 kg m^{-1} (2.7 kg m^{-2}). Harvesting was performed at monthly and bi-monthly intervals. Sampling was performed following the same procedure as in experiment 1, with the exception that all biomass harvested from each rope constituted one sample. The study was initiated in November 2000 and terminated beforehand in late January 2001 due to heavy mussel infestation.

Growth of vegetative and spore thalli; the role of reproductive status and coalescence

Coalescent and non-coalescent plants were collected from ropes with spore-seeded *Gracilaria*. Apical thalli fragments (8 cm) were collected from each of these plants. Coalescent thalli were identified as plants with multiple axes morphology emerging from the basal disc. Non-coalescent were identified as plants with single axis morphology following the description of Santelices et al. (1999). Very few non-coalescent plants were found. After examination using a stereomicroscope, all fragments were divided into vegetative, tetrasporic or cystocarpic life stages. Apical thalli fragments of the collected *Gracilaria* from the twined ropes were studied and were treated in the same way as the thalli originating from spores. This allowed for comparison of the growth potential of spore and vegetative derived thalli under controlled laboratory conditions. All fragments were individually marked with coloured tapes and weighed before being randomly placed in seven cultivation bottles, each containing 15 fragments. The thalli were cultivated at 15 °C and 53 μmol m^2 s^{-1}, with a photoperiod of 12/12 h, for 30 days. Provasoli was used as the cultivation medium (Provasoli 1968), and was changed weekly during the cultivation period. The pH, salinity, and temperature were controlled and measured on a weekly basis. At the end of the cultivation period the fresh weight and length of all the apical fragments were measured.

Statistics

Biomass production data and SGR from field studies were statistically analysed using a two factorial ANOVA analysis (STATISTICA software). The data were then analysed using Fisher's LSD-test for comparison of means (Sokal and Rohlf 1998). Data were tested for homogenous variance using Cochran's test. If necessary, data were transformed using $1/y$ transformation. The laboratory data were tested statistically by using two factorial ANOVA (Systat 5.2.1) and homogenous variances were tested using Tukey's test.

Results

Experiment 1: Comparison of two suspended cultivation methods

Mean production varied between 0.22 and 0.26 kg m^{-1} (approx. 0.6 kg m^{-2}) after 1 month for all sites, except spore-seeded ropes situated near the fish cages. These ropes produced 0.15 kg m^{-1} (0.5 kg m^{-2}), which was significantly lower compared to twined ropes at the same site ($p < 0.05$) and spore-seeded ropes at 300 m distance ($p < 0.05$)(Figure 5).

Considerable biomass was lost from all spore-seeded ropes after the first month resulting in low density and patchy cover of the ropes. There was almost no production to harvest on these ropes the following months and therefore no additional yield was accumulated (Figure 5). Small *Gracilaria* fronds observed on the ropes were left to grow. No production was observed in early spring (August and September) and the experiment of the spore-seeded ropes was

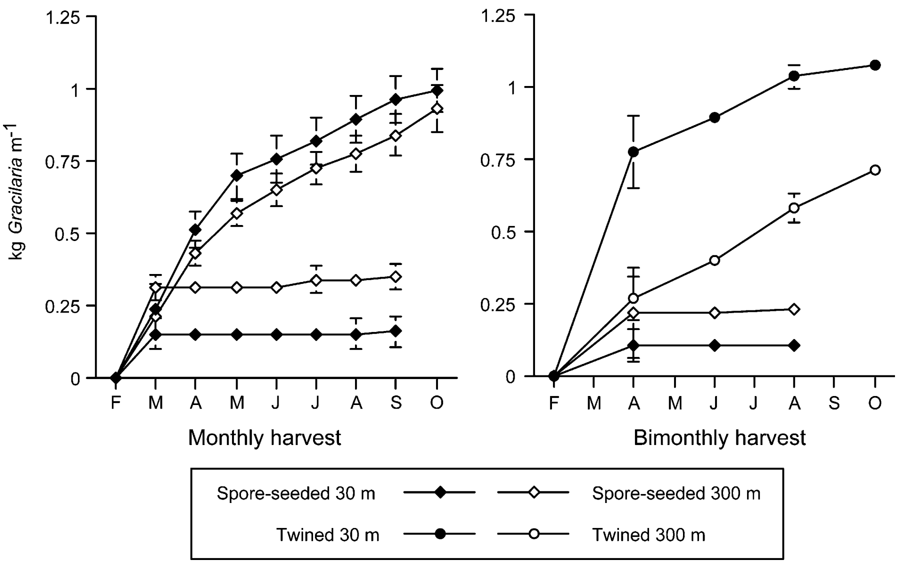

Figure 5. The accumulated yield harvested monthly (left) and bimonthly (right) (±SE, $n = 8$) of *Gracilaria chilensis* (ww) on spore inoculated and twined ropes at 30 m and 300 m from fish cages during the period of February–October 2000 (Epiphyte weight excluded).

stopped. By then, epiphytic algae, bryozoans and blue mussels heavily infested the ropes.

The twined rope experiment continued until October. No significant difference in production could be found between cultivation sites (Figure 5). By the end of the experiment epiphytes and especially epifauna, such as bryozoans, infested the twined ropes making the thalli stiff and unproductive. Some discrepancies in the tightness of the twined ropes were detected. Loose twined ropes suffered from biomass loss, which did not occur if the ropes were tightly twined.

The bi-monthly harvest showed the same pattern as the monthly harvest (Figure 5). The production at the first bi-monthly harvest of the twined ropes, 30 m from the fish cages, was significantly higher than all of the other ropes and sites (twined 300 m $p < 0.01$, spore-seeded 30 m $p < 0.001$ and 300 m $p < 0.01$) (Figure 3). The bi-monthly yield from twined ropes, after 2 months, was higher than the accumulated yield from monthly harvesting. However, the effect of bimonthly harvesting declined after two harvests, and the overall accumulated yield after eight monthly and four bimonthly harvests was similar (Figure 5).

Experiment 2: Two types of thalli tested using the twisted-rope cultivation method

The growth rate observed in spore-seeded thalli at 30 m distance from the fish culture was 2.32% ± 0.23 during the first month of cultivation (Figure 6). This was not significantly different from the control site (2.10% ± 0.66). A similar relationship was found for collected thalli (1.83% ± 0.6 and 1.62% ± 0.4).

The mean SGR for all treatments ranged between 0.7 and 1.4%, with large variation within groups, after 2 months. No significant differences between thalli types or sites were found. Bimonthly production was not significantly different between thalli types or between sites and the epiphytic load was high overall. The study was stopped after the harvest in January due to heavy mussel settlement, covering almost all *Gracilaria*. No significant differences in epiphytic load were found between the different thalli types.

Growth of vegetative and spore thalli and the role of reproductive status and coalescence

Gracilaria from twined ropes with collected thalli had a mean SGR of 4.8 ± 1.4% under controlled conditions. This was significantly higher compared to the 2.4 ± 0.9% ($p < 0.001$) of the spore-seeded thalli (Figure 7a). The thalli from the twined ropes were homogenous in life stages, showing no signs of reproductive structures. The spore-seeded thalli were, however, highly heterogeneous, consisting of vegetative, cystocarpic and tetrasporic life stages. The

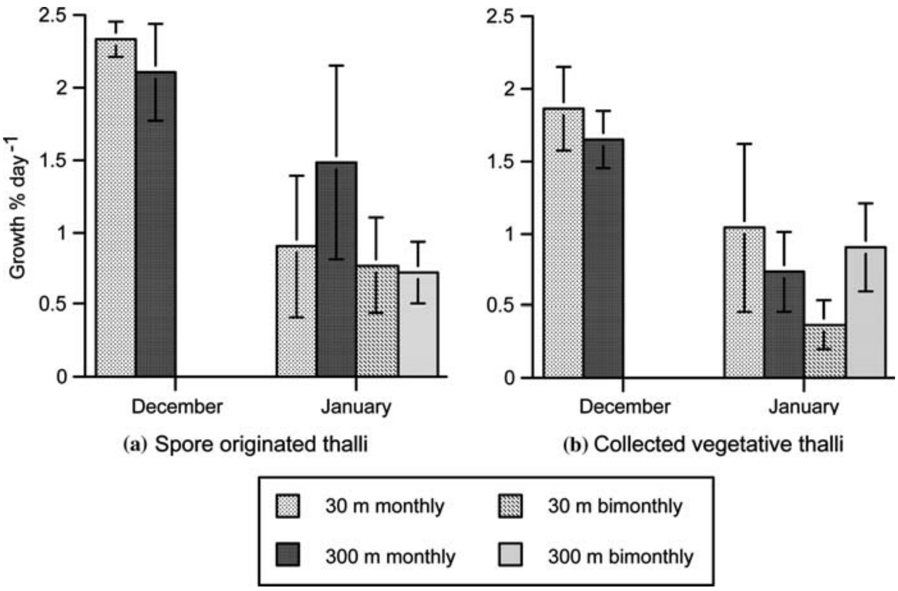

Figure 6. The mean specific growth rate (SGR) ($\pm$SE, $n = 4$) of two different types of thalli, spore originated (a) and collected (b) of *Gracilaria chilensis* cultivated and harvested at two different distances from a salmonid farm in December and January 2000–2001. (Epiphyte weight excluded).

growth rate of the vegetative phase was (2.9 ± 0.9%) not significantly different from the tetrasporic stage (2.1 ± 0.7%) or cystocarpic stage (2.4 ± 1.0%) (Figure 7b).

The spore-seeded ropes showed that over 99% of the *Gracilaria* plants attached to ropes had a coalescent morphology. This evidence indicates the higher survivorship of the coalescent plants. However, no difference in growth rate was found between coalescent thalli (2.4 ± 0.04%; $n = 98$) and non-coalescent thalli (2.3 ± 0.3%; $n = 5$).

Discussion

When the spore-seeding method was developed by Alveal et al. (1997) it showed a high production potential with 6.5 kg m^{-1} after 15 months of cultivation. Nevertheless, the method was only tested under protected estuarine conditions. The biomass losses in the present study indicate that the culture method may not be suitable for the exposed open coastline of Chile. As *G. chilensis* lacks a developed attachment disk, all methods for cultivation are based on its development of an underground thalli system in soft substrate (McLachlan and Bird 1986; Buschmann et al. 1995). This morphological character is preventing successful cultivation on relatively hard substrates such

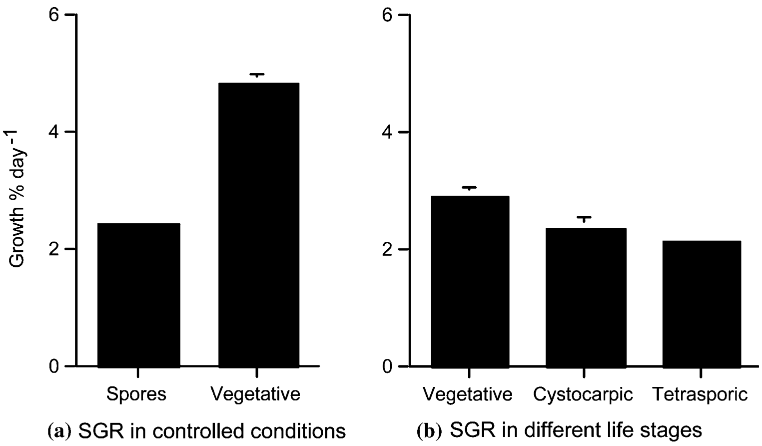

Figure 7. (a) The difference in SGR (±SE) between thalli apices from spore-seeded *Gracilaria chilensis* (SGR 4.8 ± 1.4% day^{-1}; $n = 75$) and collected (SGR 2.4 ± 0.9% day^{-1}; $n = 102$) cultured in controlled laboratory conditions ($p < 0.001$). (b) SGR for three different life stages (±SE) of *Gracilaria chilensis* found within the spore seeded material cultivated in controlled laboratory conditions (ns); vegetative (2.9 ± 0.9% day^{-1}; $n = 29$), cystocarpic (2.4 ± 1.0% day^{-1}, $n = 30$) and tetrasporic (2.1 ± 0.7% day^{-1}; $n = 44$).

as culture ropes. Ropes that happened to be loosely twined were also more vulnerable to exposure; however, this loss was significantly less than on the spore-seeded ropes. Generally, the twined ropes became tight and firm as the bushy thalli developed. The production from twisted ropes in November–December was 1.34 kg m^{-2} $month^{-1}$ at the control site, which is similar to the production obtained with the same technique by Dr. Westermeier (Universidad Austral, Chile, pers. comm.) and is significantly higher than intertidal bottom cultures (Buschmann et al. 1995). Even though the potential for production is high, suspended culture is not being practised on a commercial basis. This is probably due to the relatively high capital investment needed for ropes, frames, anchors, buoys and boats. There is less pressure to exploit new farming areas, as the market price for *Gracilaria* has decreased in the last decade. If demand should increase, suspended culture may constitute the only practical farming method as no more shallow areas are available for expansion. The development of rope culture systems for bioremediation purposes could be a solution for the salmon industry to become less environmentally degrading (Troell et al. 1997, 2003) and would increase the production of *Gracilaria*.

Under controlled conditions, the fertile life-stages (cystocarpic and tetrasporic) of the spore-originated thalli have lower SGR compared to the sterile (vegetative) phase. This confirms reduction in thalli growth due to extra energy costs for producing gametes and spores (Kain and Destombe 1995). Spore-originated thalli have a higher polymorphism compared to vegetative thalli, which could be the result of different genotypes. This could generate a wide variety of characteristics and as a result a higher capacity for adapting to changes in environmental conditions (Santelices et al. 1999). Collected thalli are known to be characterised by more morphological and genetic homogeneity, resulting in a homogeneous high growth under certain favourable conditions. This homogeneity may restrict the ability to cope with changes in environmental conditions. The absence of higher growth by the polysporic coalecent thalli, compared to those collected under controlled conditions, as well as in field, may be a result of the capability of *Gracilaria* to modify their growth pattern within the clonal population, by intraclonal variation (Santelices 2001)

The study of coalescence, and its implications on *Gracilaria* cultivation, is still in its infancy (Santelices 2001). Suggested implications, such as, higher growth and resistance to mechanical dislodgement (Muñoz and Santelices 1994; Santelices et al. 1999; Santelices 2001) were not detected in controlled conditions or in open water cultivation. The suggested higher epiphytic resistance of coalescent spore-seeded thalli (Muñoz and Santelices 1994; Santelices et al. 1995; Alveal et al. 1997; Santelices 2001) was not found in this study. Those findings are in accordance with Glenn et al. (1996, 1998) using spore-seeded *Gracilaria* ropes. The magnitude of epiphytic load may vary depending on biological and environmental characteristics, as well as seasonality. The epiphytic load found in the present study may have masked differences in resilience between the two thalli types. Spore-originated thalli could be a convenient way for continuous restocking of twisted cultivation ropes. Nevertheless, extra costs needed for hatchery facilities and nursery grounds may prevent the success of such a practice. If spore-originated thalli are to be used, aspects like agar quality need also to be studied, as increased polymorphism may induce variations that do not maintain a continuous high quality. To increase the performance of the rope cultures even further, net tubes could be introduced. These have been suggested for culturing seaweeds in coastal areas (Smit and Bolton 1999; Zertuche-González et al. 2001). Advantages associated with the use of net tubes would be less biomass loss and easier harvesting and restocking. However, the spore-seeding system still offers the easiest seeding method and furthermore, the cost of the ropes is significantly lower than net tubes. Perhaps a combination of the two methods would be advantageous. Spore-seeded ropes could be used for the first outgrowth in protected bays (nursery areas), while net tubes could be used for continued production in open waters.

The accumulated monthly yield for twined ropes cultivated at 30 m distance from the fish cages was 30% less after two harvests compared to the bimonthly harvesting (Figures 5 and 6). Kuschel (unpublished results) found that the accumulated yield in a Chilean bottom cultivation almost doubled if harvested once every 3 months, due to the removal of the fast growing apical parts of the thalli. Therefore, harvest frequency seems to be important and needs to be adjusted for optimisation of production. However, harvesting must be performed frequently enough to avoid large thalli fragmentation due to weight and epiphytes (Glenn et al. 1998) and also to avoid nutrient and light limitation in large and dense cultures.

Integrated seaweed aquaculture systems have been suggested as a possible solution for securing an increasing and environmentally sound production of future supply of fish and seafood (Troell et al. 1997, 1999; Naylor et al. 2000; Chopin et al. 2001; Troell et al. 2003). Results from this study indicate that integrated cultures using seaweeds may not be that simple and that the choice of culture method may be crucial. The growth rates were not significantly higher in *Gracilaria* cultivated near the cages, even though a trend for increased growth could be seen for both thalli types near the fish cages (Figures 3 and 4). This was surprising as a previous study carried out at the same site and time of the year. Troell et al. (1997) observed a 40% higher growth rate near the cages. The absence of a strong positive effect upon growth from the fish farm could be due to an increased overall nutrient concentration of the study area during the present study, as this was not monitored. The overall growth rate in the study by Troell et al. (1997) was substantially higher (up to 7%) compared to the present study, indicating more favourable conditions for seaweed growth i.e. light conditions and temperature. These parameters are often preponderant for *Gracilaria* growth in nutrient enriched water (De Casabianca et al. 1997). This discrepancy in the results from the two integration culture studies stress the need for a more detailed understanding of the biological and biochemical processes in open water seaweed integrated systems.

Nevertheless, *Gracilaria* cultivated under integrated conditions is productive even during winter months. This is not observed in the traditional bottom cultures due to harvests and storms removing the biomass (Buschmann et al. 1995). Moreover, the continuously stocked ropes give the possibility for enhancing *Gracilaria* cultivation in integrated suspended conditions. No evidence was found for *Gracilaria* thalli loosing their productivity with age. Cultivation of ropes need careful harvest management and also regular restocking, mainly due to epifaunal infestation. Inoculation of spore originated thalli on ropes could help to maintain high productivity. Retamales et al. (1994) demonstrated that intensive tank cultivation of *Gracilaria* could be maintained without any renewed inoculum for 3 years and with no production loss.

The future success of integrated cultivation in Chile depends to a large extent on the development of easily manageable, economically and ecologically sound cultivation methods. Spore inoculated ropes of *Gracilaria chilensis* may be useful for continuous supply of stocking material for such cultivation methods; however, its performance in open coastal water is limited due to biomass loss. The vegetative seeded ropes described in this study show a significant advantage over previous studies using algal bundles.

However, the cultivation techniques still need to be developed and productivity needs to be improved, in order to outweigh costs for nursery areas, labour, etc., before the suspended seaweed culture will become a practised cultivation technique in integrated open seawater systems.

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