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Settlement, Growth and Survival of the Donkey's Ear Abalone *Haliotis asinina* (Linne) in Response to Diatom Diets and Attachment Substrate

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The effect of feeding four diatom diets (*Amphora* sp., *Navicula ramosissima*, *Amphora* sp. + *N. ramosissima*, and mixed diatoms) and two attachment substrates (PP+CCA: polyvinyl plates with crustose coralline algae; PP-CCA: polyvinyl plates without crustose coralline algae) were determined for seed production of abalone, *Haliotis asinina*. On day 5, significantly higher number of larvae settled on PP+CCA fed with mixed diatoms followed by *Amphora* sp., *N. ramosissima*, *Amphora* sp. + *N. ramosissima*, and abalone larvae reared on PP-CCA fed with *N. ramosissima* only. Fewer larvae settled on PP-CCA fed with *Amphora* + *N. ramosissima*, *Amphora* sp. and mixed diatoms. The size of abalone juveniles from PP-CCA was significantly bigger compared with juveniles measured from PP+CCA. However, the number of juveniles harvested from tanks provided with PP+CCA was higher compared with tanks with PP-CCA. This study has shown that crustose coralline algae favored the settlement of *H. asinina* larvae and inoculation of diatom slurry is necessary to provide sufficient food for the growing larvae.

Key Words: abalone seed production, benthic diatom, biofilm, settlement inducer

INTRODUCTION

Benthic diatom films growing on plastic plates have been used as settlement substrata for postlarval abalone due to their extracellular polymeric substances (Hoagland et al. 1993) which are considered as the principal food for post-larval and early juvenile abalone (Kawamura and Takami 1995; Kawamura 1996). In the abalone hatchery of the Southeast Asian Fisheries Development Center, Aquaculture Department (SEAFDEC/AQD) postlarvae and juveniles are grown on natural population of diatoms attached to corrugated polyvinyl plates without adopting the open pond batch system used in culturing monospecies of diatoms. This method does not assure a consistent supply of desired diatom species needed by the abalone larvae due to the presence of unwanted species (Norman–Boudreau et al. 1986). Also, the use of mixed species of diatom can not maintain the desired quantity of food which is important to growing postlarvae. To address this problem, mass culture methods of the locally isolated *Amphora* sp. (de la Peña 2007) and *Navicula ramosissima* and mixed diatoms were developed. *N. ramosissima* was considered as an additional diet due to its small size and it is one of the commonly used diatoms in abalone hatcheries (Kawamura and Takami 1995; Kawamura et al. 1998; Roberts et al. 1999; Daume et al. 2000; Gallardo and Buen 2003).

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The use of corrugated polyvinyl plates with grown crustose coralline algae (CCA) as settlement substrate has been practiced in hatcheries for other abalone species. No study has yet been conducted to evaluate its effect on the settlement rate of the tropical abalone *H. asinina*. In natural habitats, abalones prefer to settle on CCA due to the presence of chemical cues, biofilm, and bacteria (Morse et al. 1984; Morse and Morse 1984). However, growing CCA on new corrugated plates requires three to six months before they can be used in the hatchery. This lag time of growing CCA on settlement plates cannot cope with the increasing demands for abalone juveniles in stock enhancement program. To address this problem, we tested the hypothesis that larvae of the abalone *H. asinina* prefer to settle on corrugated polyvinyl plates with growing coralline red algae compared with plates without coralline red algae.

As part of SEAFDEC/AQD's refinement of hatchery rearing techniques, the effect of mixed diatom diet on abalone settlement and postlarval growth needs to be investigated in comparison with monospecific diatom diets. The study of Gallardo and Buen (2003) on evaluating different biological films for *H. asinina* was done only *in vitro* (petri dishes). Their result has to be verified under conditions of commercial hatchery scale. The present study was done in pilot hatchery production scale (1000 L fiber glass tank) for a period of 90 d. This period of rearing is considered as one hatchery cycle to produce 0.5 to 1.0 cm abalone seed. This study was aimed to standardize the hatchery feeding protocol by comparing four diatom diets (*Amphora* sp., *Navicula ramosissima*, *Amphora* sp. + *N. ramosissima*, and mixed species of diatoms) and two attachment substrates (PP+CCA: polyvinyl plates with crustose coralline algae; PP-CCA: polyvinyl plates without crustose coralline algae) on the settlement, growth, and survival of *H. asinina* larvae.

MATERIALS AND METHODS

Preparation of Diatom Slurry and Settlement Plates

Pure stock cultures of *Amphora* sp. (3.0 x 11-13.0 µm) and *N. ramosissima* (5 x 10.0 µm) were obtained from the larval food laboratory of SEAFDEC/AQD. The monospecific diatom starters were maintained in controlled conditions (de la Peña 2007) using F/2 medium (Guillard and Ryther 1962). The diatoms were cultured from stock to larger culture vessels up to 5000 L using the multi-step batch culture method. Each species was harvested at its exponential growth phase before transferring to a bigger scale of culture. Initially, 300 L of diatom starter was inoculated into the 5000 L concrete tank and was filled with 700 L of seawater which passed through a sand-filter and a 5 µm rated filter bag. The culture was vigorously aerated from the bottom of the tank using a perforated polyvinyl blue pipes that serve as the aeration line. The cultures were enriched using the modified Commercial 11 medium (Renaud et al. 1991) composed of technical and agricultural grade reagents (ammonium sulfate 21-0-0, 150.0 mg L^{-1}, urea 46-0-0, 7.5 mg L^{-1}, super phosphate 16-20-0, 25 mg L^{-1}, $FeCl_3.6H_2O$, 5.0 mg L^{-1}, Na_2EDTA, 5.0 mg L^{-1}, sodium silicate 15.0 mg L^{-1}). After 3 d of culture, additional seawater and fertilizer were added to fill-up the tank to full capacity. The tank was set up with two bamboo poles in parallel that each held 50 pieces of 122 cm x 114 cm corrugated plastic sheets to induce the growth of benthic diatom on the plates. After another 3 d, the diatoms were harvested using a soft paint brush and the slurry was stocked in a bucket in preparation for inoculation in the abalone settlement tank.

Mixed diatom starter was obtained from SEAFDEC/AQD concrete tanks where seaweeds were cultured. The diatoms that attached to the sides of the concrete tanks were scraped using a soft paint brush and directly used as starter in five tons concrete tanks. Similar to the steps used in pure cultures of diatoms, the tank was set up with two bamboo poles in parallel that each held 50 pieces of 122 cm x 114 cm corrugated plastic sheets to induce the growth of benthic diatom on the plates. The tank was directly filled up to 5000 L of seawater and enriched with Commercial II fertilizer that was prepared in the laboratory. After 4 d (when mixed diatoms had started to attach to the plates), flow through of seawater was initiated until the culture plates were covered with growth of mixed species of diatom. The diatoms that attached to the plastic sheets and concrete tanks were harvested using a soft paint brush and were concentrated to 25 µm net bag. The bigger size diatoms were removed by using a 60 µm net bag. Two-thirds of the diatom slurry was used as feed for abalone larvae while the remaining one-third was returned to the tank as a starter for the next batch of culture. One culture cycle ran for three weeks and the mixed diatom starter was replaced every cycle. The dominant diatom species in the plate were identified and their percentage cover was estimated. The mixed species of diatoms was composed of *Navicula* spp. (65%), *Melosira* spp. (14%), *Amphora* spp. (6%), *Nitzschia* spp. (6%), *Grammatophora* spp. (2%), and *Cocconeis* spp (1%).

Eight 1000 L rectangular fiber glass tanks were used as the rearing vessels. Each tank was set up with two parallel bamboo poles with 25 pieces of 122 cm x 114 cm settlement plates (= 50 plates/tank) each pole. Four tanks were

installed with corrugated plates with CCA (PP+CCA) and the remaining four without CCA (new plates, PP-CCA). The plates with CCA (PP+CCA) were old settlement plates with encrusting coralline algae identified as *Mesophyllum* sp. on the top and *Hydrolliton samoense* at the bottom of the pink crust. To grow the CCA, new plates were inserted in between old plates with growing juveniles to allow the carpospores or gametes of the coralline red algae to attach to the new plates. The presence of abalone mucus in the plates induces the settlement of the spores. After 3-6 mo of exposure, new patches of pink crust appeared on the plate. Each rearing tank was given one type of diatom diet. To induce the diatoms to attach to the plates without CCA, the smooth surface was scratched using a number 60 silicon carbide resin bond paper (Atlas, English Abrasives & Chemicals Limited). The tanks were inoculated with diatom slurry 5 d before stocking the larvae. Additional inoculation of diatom slurry was done every 3 d thereafter.

Abalone Larval Rearing

Concentrated abalone pre-veliger larvae were obtained from the broodstock facility of SEAFDEC/AQD abalone hatchery. The density of abalone larvae was estimated by counting the larvae in 10 one-ml sub samples drawn from the whole population. The trocophore larvae were collected by siphoning into a 100 L plastic bucket lined with 45 μm mesh size net to regulate the slow flow through of UV irradiated seawater for washing the larvae.

The larvae were stocked and distributed to the rearing tanks at a density of 250 per liter. Mild aeration using two air stones was provided on each tank. A 36-watt Toshiba cool-white fluorescent lamp (Watt Brighter, Thailand) was provided for every two tanks during night time to reduce respiration of diatoms. Static water system was maintained during the first 10 d of rearing. After 10 d of incubation, flow through seawater system (3-8 L per min) was initiated. The larvae were fed with diatom slurry every 3 d thereafter for a period of 90 d. The flow of seawater was stopped for 1 h after feeding. Temperature and salinity were set at 27-29°C and 32-33 g L^{-1}, respectively.

The diatom species tested differed in size and therefore feeding concentrations were determined according to their respective average dry weights. The dry weight of each species was determined using the methods of Brown & Jeffrey (1995). The dry weight of *N. ramosissima* was 0.000709 μg $cell^{-1}$, while that of *Amphora* sp. was 0.000444 g $cell^{-1}$. The dry weight of mixed diatom was 0.0003284 μg $cell^{-1}$. The amount of food was set at 10 μg enough to develop a thin diatom film during the initial stage of rearing. Hence, to feed the same amount of dry mass in all treatments, 14,000 cells mL^{-1} of *N. ramosissima*, 22,000 cells mL^{-1} of *Amphora* sp. and 30,000 cells mL^{-1} of mixed diatom were considered as feeding concentrations. The treatment that used the combination of *Amphora* sp. and *N. ramosissima* was given a 50:50 feed ration (7,000 cells mL^{-1} *N. ramosissima*: 11,000 cells mL^{-1} *Amphora* sp.). The cell concentration of each species was determined by direct counts using a haemocytometer and a compound microscope (Martinez et al. 1975). The evaluation of cell concentration per area on the plate (cm^{-2}) was based on the methods of de la Pena (2007).

Sample Collection and Statistical Analysis

The number of settled postlarvae was monitored every 5 d for a period of 15 d. Two replicate plates were removed from the tanks each time and the settled larvae were dislodged from the plate using a soft paint brush. The collected larvae were placed in a small basin and were concentrated in a 45 μm net and placed in plastic sampling bottles containing 50 ml seawater. The larvae were examined under a SMZ-10 Nikon stereoscopic microscope (Nikon Corporation,Japan). The dead ones were excluded from counting. Growth of juveniles was monitored by measuring shell length (SL) of 30 individuals after 60 d and every 15 d thereafter using a Vernier caliper. The experiment was terminated after 90 d of rearing and the total number of juveniles that survived on each tank was recorded.

Statistical analysis was carried out using a 2 x 4 factorial design to test the effects of diatom diets and attachment substrate on the settlement, growth, and survival of abalone larvae. Number of settled larvae and juveniles that survived was transformed to their square root before calculating for analysis of variance (ANOVA). Differences between means of replicate analysis were tested for significance (P>0.05) with the Duncan's multiple range test (DMRT) (SPSS Version 11).

RESULTS AND DISCUSSION

Table 1 shows the postlarval attachment on day 5 and post settlement survival on day 10 and day 15. Larval attachment on substrate with grown CCA (PP+CCA) fed with mixed diatoms (25.7 postlarvae per plate), *Amphora* sp. (24.0 postlarvae per plate), *N. ramosissima* (20.5 postlarvae per plate) and *Amphora sp + N. ramosissima* (15.0 postlarvae per plate) were significantly higher (p<0.05) than in substrate without CCA (PP-CCA) fed with *Amphora sp + N. ramosissima* (10.5 postlarvae per plate), *Amphora* sp (6.5 postlarvae per plate), mixed diatoms (6.5 postlarvae per plate) except for *N. ramosissima* fed that showed a comparable number of settled larvae (19.5 postlarvae per plate). The higher larval attachment on plates with CCA is due to the biofilms

Table 1. Number of settled *Haliotis asinina* larvae fed with four diatom diets ((*Amphora* sp., *Navicula ramosissima*, Amphora sp. + N. ramosissima, and mixed diatoms) and two attachment substrates (PP+CCA: polyvinyl plates with crustose coralline algae PP-CCA: polyvinyl plates without crustose coralline algae).

Diatom diets		Substrate	Culture Period		
			Day 5 (Post-larvae plate^{-1})	Day 10 (Post-larvae plate^{-1})	Day 15 (Post-larvae plate^{-1})
Amphora sp.		PP+CCA	24.0 ± 2.0^{a}	33.5 ± 3.5^{a}	20.0 ± 10.0^{a}
N.ramosissima		PP+CCA	20.5 ± 6.5^{a}	4.5 ± 1.5^{a}	63.5 ± 24.5^{a}
Amphora sp.	+	PP+CCA	15.0 ± 7.0^{a}	34.5 ± 12.5^{a}	26.0 ± 1.0^{a}
N.ramosissima					
Mixed diatoms		PP+CCA	25.7 ± 2.5^{a}	8.0± 6.0^{a}	5.0 ± 0.0^{a}
Amphora sp.		PP+CCA	6.5 ± 0.5^{c}	10.0 ± 5.0^{a}	39.5 ± 16.5^{a}
N.ramosissima		PP+CCA	19.5 ± 0.5^{a}	3.0 ± 1.0^{a}	3.5 ± 0.5^{a}
Amphora sp.	+	PP+CCA	10.5 ± 3.5^{b}	28.5 ± 10.5^{a}	144.5 ± 107.5^{a}
N.ramosissima					
Mixed diatoms		PP+CCA	6.5 ± 3.5^{c}	25.0 ± 0.2^{a}	38.5 ± 28.5^{a}

Values are mean ± SEM (n=2).
Mean values within culture period having the same superscripts are not significantly different (P>0.05).

consisting of bacteria, diatoms, and other microorganisms embedded in the CCA crust. The macromolecules and chemical cues that are excreted by coralline algae *Mesophyllum* sp. and *Hydrolithon samoense* provided an additional cue that favored settlement. Larvae of several abalone species prefer to settle on non-geniculate coralline red alga (Corallinales, Rhodophyta) in the natural environment (Daume et al. 1999). Larval settlement and metamorphosis are shown to be triggered by a class of chemical inducers related to phycobiliproteins found in the extracts of coralline red algae (Morse et al. 1984; Morse and Morse 1984).

Among the diatom diets tested, larvae provided with mixed diatom resulted in highest settlement due to the presence of *Navicula* spp. (65%). It is classified as a prostrate type A diatom which can be ingested easily due to its low adhesive strength (Kawamura et al. 1998). Prostrate diatoms are effective in forming flat diatom community which is the preferred substrata for settlement and metamorphosis of larval abalone (Kawamura and Kikuchi 1992). This type of diatom community is effective in releasing the extra cellular substances needed by the newly settled larvae. The other types of diatom communities (Types C, D, E, F, G, and H) grow as upright colonies are preferred by juveniles and adult gastropods. This result is similar with the study of Gallardo and Buen (2003) which showed that *Navicula* sp. supported higher settlement of *H. asinina*. *Navicula* sp. is one of the most used genera in the production of films for abalone culture (Siqueiros-Beltrones and Voltolina 2000). The low settlement of larvae in plates without CCA (PP-CCA) fed with mixed diatom could be due to the absence of chemical cue from crustose coralline alga. The presence of mixed diatom excretion alone may not be enough to induce higher settlement of *H. asinina* larvae in new polyvinyl plates. The high settlement of *N. ramosissima* in plates without CCA (PP-CCA) is due to the type of extracellular substance excreted by this species which is favorable to *H. asinina* larvae. Joouchi et al. (2007) found that *N. ramosissima* releases sugar compound products (LCA-binding sugar chain) that induce settlement in the barnacle, *Amphibalanus amphitrite*. Daume et al. (1999) observed high settlement rate when they used *N. ramosissima* on *H. laevigata.* The higher larval settlement of abalone fed with a mixture of *Amphora* sp. + *N. ramosissima* showed that mixed feeding is more effective than sole feeding. The absence of CCA on this treatment was compensated by the extracellular excretions of these diatoms that induce settlement. This result is supported by the studies of Daume et al. (2000) and Carbajal-Miranda et al. (2005) on the higher settlement of *H. rubra* and *H. rufescens* larvae.

Post settlement survival after day 10 and day 15 did not significantly differ in the two substrate types and among the four diatom diets tested. Although the number of settled larvae during the first five days was significantly different due to the presence of crustose coralline algae, this effect was overcome by the high biomass of diatom present in the plate without CCA (Figure 1). The diatom slurry added in plates without CCA had multiplied faster compared with plates with CCA which provide food for the growing postlarvae. Kawamura and Kikuchi (1992) found highest settlement rate of the abalone *H. discus hannai* when diatom density was high. The density of diatom is one of the most important factors to be considered in post-larval rearing (Suzuki

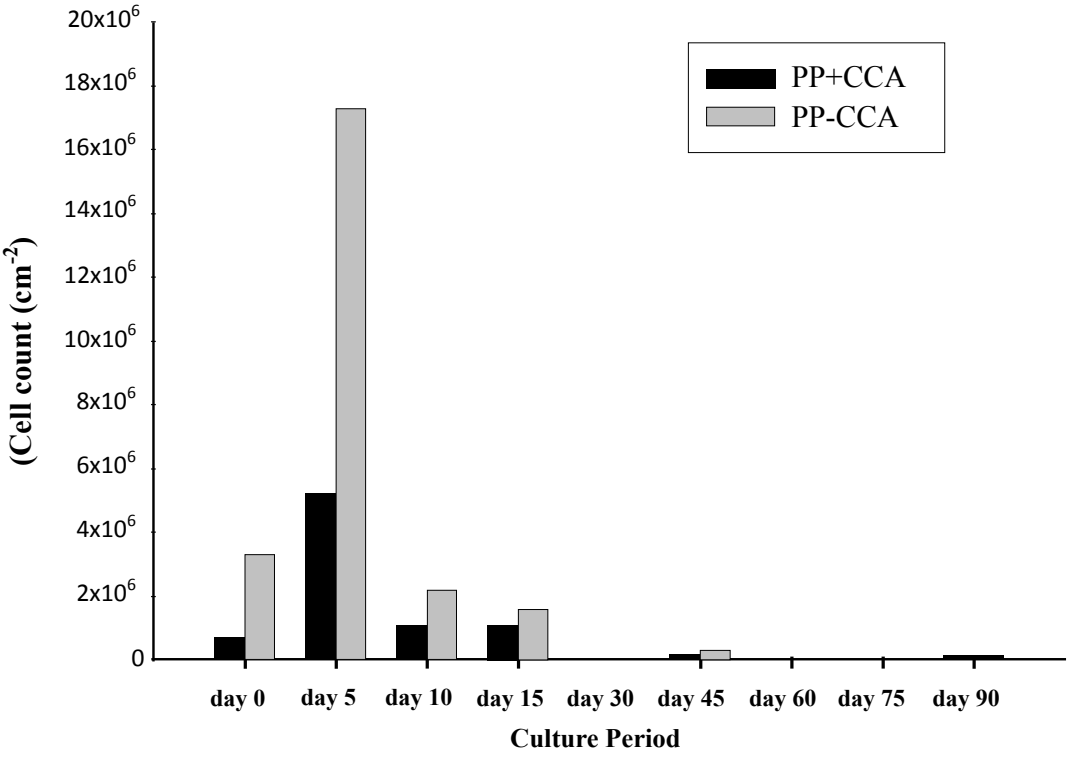

Figure 1. Total biomass of benthic diatoms in two attachment substrates (PP-CCA: polyvinyl plates with crustose coralline red algae; PP-CCA: polyvinyl plates without crustose coralline red algae) for 90 days rearing period.

et al. 1987; Kawamura et al. 1998). Larval abalones increase feeding rate as they grow (Roberts et al. 1999; Searcy-Bernal et al. 2001). During the earlier stage of rearing, the density of the diatom species tested was not sufficient to induce larval settlement but on the later stage of rearing it had multiplied and the requirement of settled larvae was met.

The size of abalone juveniles on day 60 to day 90 is shown in Figure 2. Regardless of diatom diets, the size of juveniles measured from plates without CCA (PP-CCA) were remarkably bigger (P<0.05) on day 60 (0.37 cm), day 75 (0.65 cm). and day 90 (0.92 cm) compared with juveniles recorded from plates with CCA (PP+CCA) (day 60, 0.25; day 75, 0.49; day 90, 0.74 cm). The effect in size is due to the more effective recruitment of diatom biomass in plates without CCA. In tanks without CCA (PP-CCA), the plates are whiter and the light intensity (PPFD, 0.65±0.12 µmole photon m^{-2} s^{-1}) can effectively penetrate within the plates that enhanced diatom growth. Whereas, in tanks with CCA (PP+CCA), the plates are blackish (older plates) and covered with CCA and lesser light intensity (PPFD, 0.35 ± 0.03 µmole photon m^{-2} s^{-1}) can penetrate within the plate which prevent the multiplication of the diatoms that attached to the plates and crust of the coralline algae.

The number of juveniles harvested after 90 d of rearing is shown in Figure 3. Significantly higher number of juveniles were harvested from tanks with PP+CCA, (1,356 juveniles) compared with tanks with PP-CCA (837 juveniles) (p<0.05). High survival was contributed by the higher settlement rate during the early stage of rearing. The tanks with PP-CCA fed with mixed diatom and a combination of *Amphora* sp. and *N. ramosissima* resulted in high survival at 1,630 and 1,400 juveniles, respectively. Feeding the larvae with several types of diatoms can provide the required nutritional value of the growing postlarvae (Brown and Jeffrey 1995; Carbajal-Miranda et al. 2005; Gordon et al. 2006).

In conclusion, this study showed that settlement, growth and survival of the tropical abalone *H. asinina* can be improved with the use of both monospecific and mixed species of benthic diatoms. The culture of mixed species of diatoms must adopt the procedures in growing the pure species to maintain the desired species. This study confirmed that the use of plates with crustose coralline algae favored settlement of *H. asinina* at the start of the rearing phase, followed by the inoculation of diatom slurry to ensure sufficient food for the growing post-larvae. Furthermore, the use of new polyvinyl plates can provide an immediate alternative substrate for rearing as long as the right diatom density is provided.

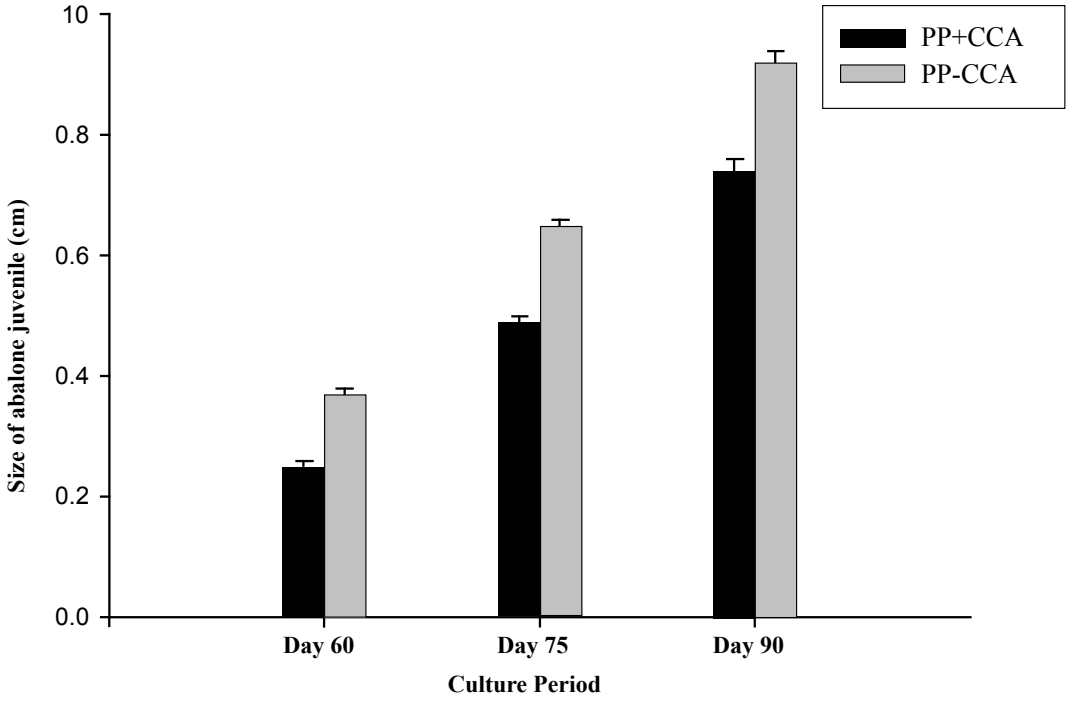

Figure 2. Size of abalone juveniles in two attachment substrates (PP+CCA: polyvinyl plates with crustose coralline red algae; PP-CCRA: polyvinyl plates without crustose coralline red algae) after 60, 75 and 90 days rearing period.

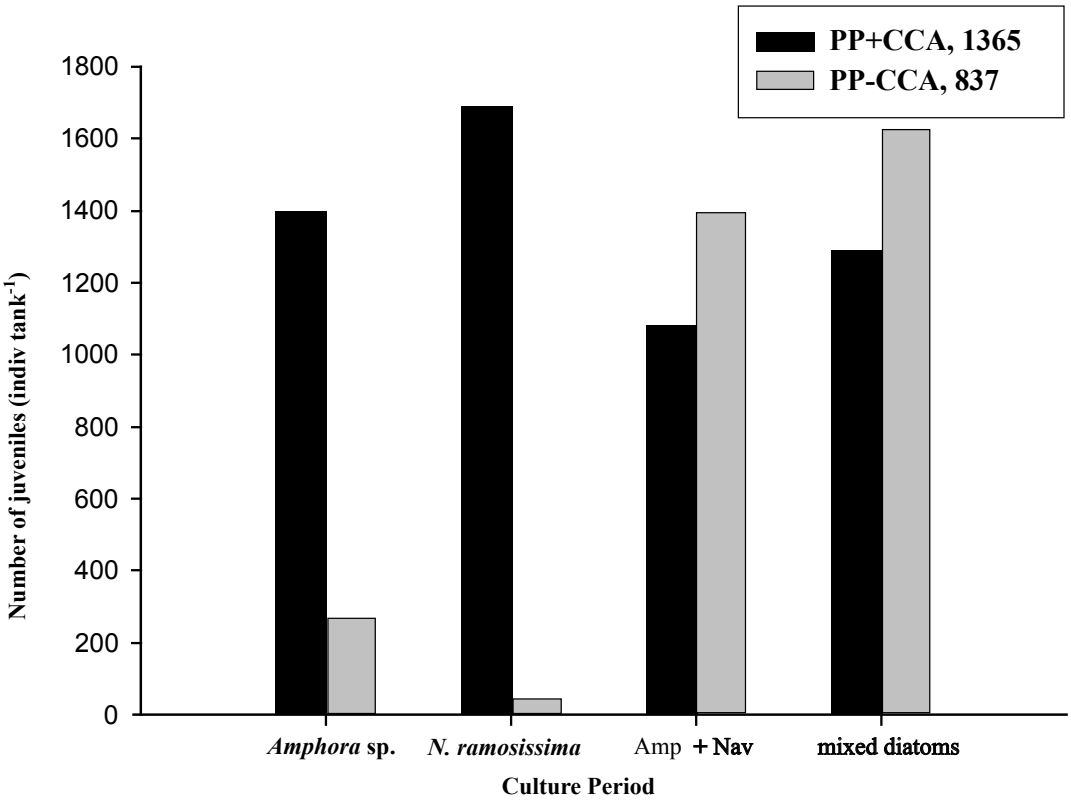

Figure 3. Number of abalone juveniles harvested fed with four diatom diets (*Amphora* sp., *Navicula ramosissima*; *Amphora* sp. + N. ramosissima and mixed diatoms) and reared in two attachment substrates (PP+CCRA: polyvinyl plates with crustose coralline red algae; PP-CCRA: polyvinyl plates without crustose coralline red algae) after 90 days rearing period.

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