Composition, size and relative density of diatoms in the stomach of 4 to 75 day-old juvenile abalone *Haliotis diversicolor* (Reeve)

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ABSTRACT

The diatom biofilm that naturally grow on polyvinyl chloride plates serve as food of postlarva and juvenile abalone *Haliotis diversicolor* Reeve, called “tokobushi” in Japanese. Composition, size and relative density of diatoms in the stomach of 4, 7, 10, 13, 17, 21, 27, 35, 50 and 75 day-old tokobushi were evaluated to characterize their diatom intake. Stomach in glycol methacrylate resin was sectioned and examined under the light microscope, then analyzed using an image processing software. The diatoms present in the stomach of tokobushi were *Thalassiosira*, *Melosira*, *Triceratium*, *Odontella*, *Asterionella*, *Licmophora*, *Thalassionema*, *Cocconeis*, *Navicula* and *Nitzschia*. Only four varieties of diatoms were observed in 4 to 10 day-old tokobushi which coincided with initial feeding. The number and size of diatoms increased in 13 to 75 day-old juvenile, which were exhibited in its exponential growth pattern. The stomach of 4 to 13 day-old tokobushi contained small-sized diatoms (<67 µm) while both small and large-sized diatoms (>123 µm) were observed in 17 to 75 day-old juveniles. Higher relative densities (8.7 – 15.8 diatom/1000 µm^2) of diatoms were documented in 4 to 10 day-old tokobushi while 17 to 75 day-old exhibited lower relative densities (1.2 – 4.2 diatom/1000 µm^2). Generally, as young tokobushi increases in size, the diatom intake increases in composition and size but density decreases with increasing size of diatom ingested.
Keywords: abalone, biofilm, diatom, glycol methacrylate, plates, stomach

INTRODUCTION

The abalone *Haliotis diversicolor* Reeve (= *H. diversicolor diversicolor*, *H. diversicolor supertexta*, *H. diversicolor aquatilis* (Geiger 1999), or ‘tokobushi’ in Japanese, are mass-produced in the hatchery for mariculture and fishery resource enhancement purposes (Alcantara and Noro 2006). Mass culture of tokobushi is done in outdoor concrete tanks provided with corrugated polyvinyl chloride (PVC) plates (hereafter referred to as plates) for settlement that have been naturally grown with biofilm of diatoms.

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Diatoms and associated microorganisms serve as food of tokobushi for the first three months in culture (Chen 1989).

The postlarval to juvenile stage is critical for survival and growth of abalone, which may be partly related to the kind and quantity of diatoms ingested as food (Day et al. 2004). Several adhesive diatoms would grow on plates but grazing rates may depend on factors such as preference and age of abalone. *Haliotis midae* postlarva prefers prostrate diatoms like *Cocconeis sublittoralis* but also ingests overstorey species like *Nitzschia palea* (Matthews and Cook 1995). On the other hand, one week-old *Haliotis rufescens* postlarva had minimal grazing on a prostrate diatom *Navicula incerta* but increased rapidly when the larvae reached 2 to 3 week-old (Martinez-Ponce and Searcy-Bernal 1998). On a commercial scale nursery culture of *Haliotis rubra*, postlarvae grow fast on the macroalga *Ulvella lens* which was further enhanced by the addition of the diatom *Navicula* sp. (Daume et al. 2004).

The gut content of young juvenile abalone in terms of diatom composition, size and relative density is a manifestation of its feeding and nutritional characteristics which has application in the aquaculture. The present paper reports on the diatom content of the stomach of 4 to 75 day-old tokobushi reared in outdoor tanks.

MATERIALS AND METHODS

One cohort of young tokobushi used in this study was hatched and cultured in the hatchery-nursery facility of the Kagoshima Mariculture Society in Tarumizu City, Kagoshima, Japan from 21 September 2004 to 21 February 2005. During hatching, the age of tokobushi is considered zero (Bryan and Qian 1998). The larvae were stocked in outdoor rectangular concrete tanks (~4 t) filled with plates naturally grown with diatom biofilm. Samples were collected randomly from 4, 7, 10, 13, 17, 21, 27, 35, 50 and 75 day-old tokobushi. Five pieces of cut-plates (~25 cm^2) with settled tokobushi were collected from each of the five tanks used for the cohort. Biofilm of diatoms that grew naturally on upright plates after 2 – 4 weeks in outdoor tanks with flow-through seawater served as the natural food of postlarval and juvenile tokobushi for about three months of culture. The different diatom populations that randomly and naturally attached on plates were the source of diatoms ingested by tokobushi in culture. The composition and density of diatoms growing on the plates were not monitored during the culture period. Continuous aeration and about eight hours flow through of seawater were provided in culture tanks.

In this study, 10 individuals of juvenile tokobushi at different ages were photographed under the stereo microscope (Nikon SMZ-U, Japan) to measure the shell length (SL) and monitor the growth rate. Microphotographs were analyzed using ImageJ: Image processing and Analysis in Java (Baggethun 2006). Another five samples were fixed in 5-10% seawater formalin for 24 – 48 hours, then transferred to 70% Ethyl Alcohol for storage. Postlarva and juvenile tokobushi were decalcified in 5% Acetic Acid, dehydrated in Ethyl Alcohol series of 70%, 80%, 90%, 95% and 100%, then infiltrated and embedded in Glycol Methacrylate (Technovit 7100®, Heraeus Kulzer GmbH, Germany) resin. Serial microtome (Yamato RV-240, Japan) sections (2 – 3 µm) were stained with Hematoxylin-Eosin or Toluidine Blue, and then photographed under a light microscope (Nikon Eclipse E600, Japan). Ingested diatoms were identified, measured and counted per 1000 μm^2 area of the longitudinally sectioned stomach to determine the composition at genera level, size in length or diameter and relative density. Using the ImageJ software, a known area (average: 1000 μm^2) in the stomach was used as the sample area of ingested diatoms (Figure 1). Identification of diatom genera were validated from diatom samples obtained from plates where tokobushi attached and grazed following the descriptions of Round et al. (1990). Relative density was calculated from the number of diatoms found in every 1000 μm^2 area in the photomicrograph of the stomach. Ingested diatoms found in the stomach of tokobushi (Figure 1) were the principal materials analyzed for the composition, size and relative density of diatom intake in this study.

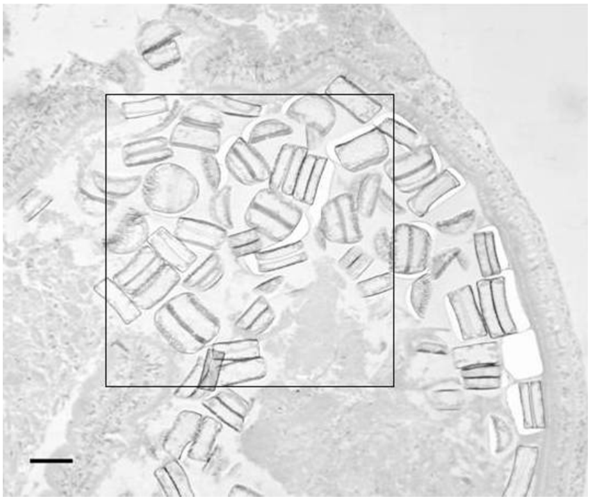

Figure 1. Photomicrograph of the stomach of a 75 day-old *H. diversicolor* showing the sample area (in a square) where ingested diatoms (shown here: *Thalassiosira*) were measured and counted using the ImageJ software; scale bar = 100 µm.

To establish the significant differences among the sizes and densities of diatom content of the digestive gut at corresponding ages, ANOVA followed by multiple comparisons was carried out using the software SIGMASTAT (Systat Software Inc., California, USA).

RESULTS

A total of 10 genera belonging to three classes, nine orders and nine families, namely *Thalassiosira*, *Melosira*, *Triceratium*, *Odontella*, *Asterionella*, *Licmophora*, *Thalassionema*, *Cocconeis*, *Navicula* and *Nitzschia* were observed in the stomach of tokobushi. These diatoms were also observed in the intestine, digestive gland and digestive caecum. *Thalassiosira* and *Melosira* were centric diatoms while the rest were pennate diatoms.

All the above diatoms were also found in the biofilm examined on PVC plates during the culture of tokobushi. However, not all diatoms were observed in the stomach of tokobushi at all ages examined (Table 1). The most commonly ingested diatoms were *Cocconeis*, *Nitzschia*, *Melosira*, *Navicula*, *Thalassionema* and *Licmophora* found in 100% to 70% of the ages examined. On the other hand, the other diatoms were only observed in 50% to 10% of the tokobushi. Moreover, 4 to 13 day-old postlarvae had ingested 4 to 6 genera of diatoms while 17 to 50 day-old tokobushi had 7 to10 diatoms in their stomach. In addition, the 4 to 75 day-old tokobushi exhibited an exponential growth pattern (Figure 2).

Table 1. Distribution of diatoms observed in the digestive gut of *H. diversicolor* at different ages in mass culture (x=present).

Diatoms	**Day-old**									
	4	**7**	**10**	**13**	**17**	**21**	**27**	**35**	**50**	**75**
Thalassiosira						x	x	x	x	x
Melosira		x	x	x	x		x	x	x	x
Triceratium					x			x		
Odontella						x		x		
Asterionella					x		x	x	x	
Licmophora	x	x		x	x	x	x	x	x	
Thalassionema		x	x	x	x	x	x	x	x	
Cocconeis	x	x	x	x	x	x	x	x	x	x
Navicula	x		x	x	x	x	x	x	x	x
Nitzschia	x	x	x	x	x		x	x	x	x

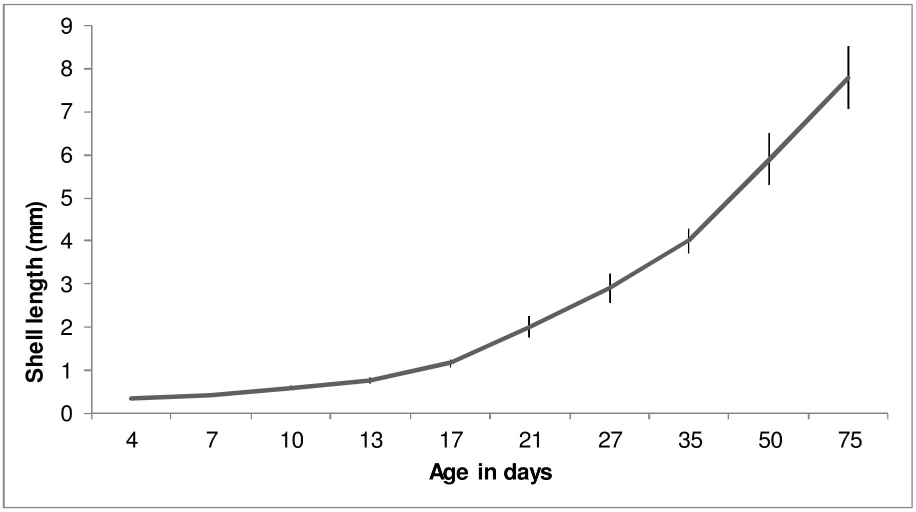

Figure 2. Growth pattern of the shell length of 4 to 75 day-old *H. diversicolor* in culture (error bar = SD).

Ingested *Cocconeis*, *Nitzschia*, *Navicula*, *Melosira*, *Odontella* and *Triceratium* were relatively small-sized diatoms ranging from 11 to 49 µm in length or diameter. On the other hand, *Thalassionema*, *Licmophora*, *Thalassiosira* and *Asterionella* were relatively large-sized ranging from 20 to 123 µm. The sizes of ingested diatoms ranged from 10 to 67 µm for 4 to 13 day-old tokobushi while 12 to 123 µm for 17 to 75 day-old (Table 2). Pooled data revealed that the size of ingested diatoms for 4 day-old was significantly smaller than those in 17, 27, 35 and 50 day-old postlarvae ($P<0.001$).

Relative densities of ingested diatoms were noted to be highest in 4, 7 and 10 day-old tokobushi which ranged from 8.7 to 15.8 diatom/1000 μm^2 of the stomach area. Such relative density was significantly different ($P<0.001$) from those in 17, 21, 27, 35, 50 and 75 day-old which ranged from 1.2-4.2 diatom/1000 μm^2 of the stomach area. Moreover, the relative density of 13 day-old (5.4 diatom/1000 μm^2) was not significantly different from those in 4 (8.7 diatom/1000 μm^2), 7 (9.7 diatom/1000 μm^2), 10 (15.8 diatom/1000 μm^2), 17 (4.2 diatom/1000 μm^2), 21 (4.5 diatom/1000 μm^2), 27 (2.5 diatom/1000 μm^2) and 50 (2.6 diatom/1000 μm^2) day-old tokobushi (Figure 3).

Table 2. Size (µm) distribution of diatoms observed in the digestive gut of *H. diversicolor* at different ages.

Diatoms	**Day-old**									
	4	**7**	**10**	**13**	**17**	**21**	**27**	**35**	**50**	**75**
Thalassiosira						58	91	116	81	123
Melosira		11	32	17	19	25	30	47	21	34
Triceratium					49			46		
Odontella								44		
Asterionella					114	99	86	112	104	
Licmophora	32	47		67	80		102	109	89	
Thalassionema		26	20	23	34	20	29	77	81	
Cocconeis	12	11	10	17	15	12	16	12	12	14
Navicula	22		17	24	19	20	25	20	23	19
Nitzschia	16	27	28	37	32	31	45	36	42	33

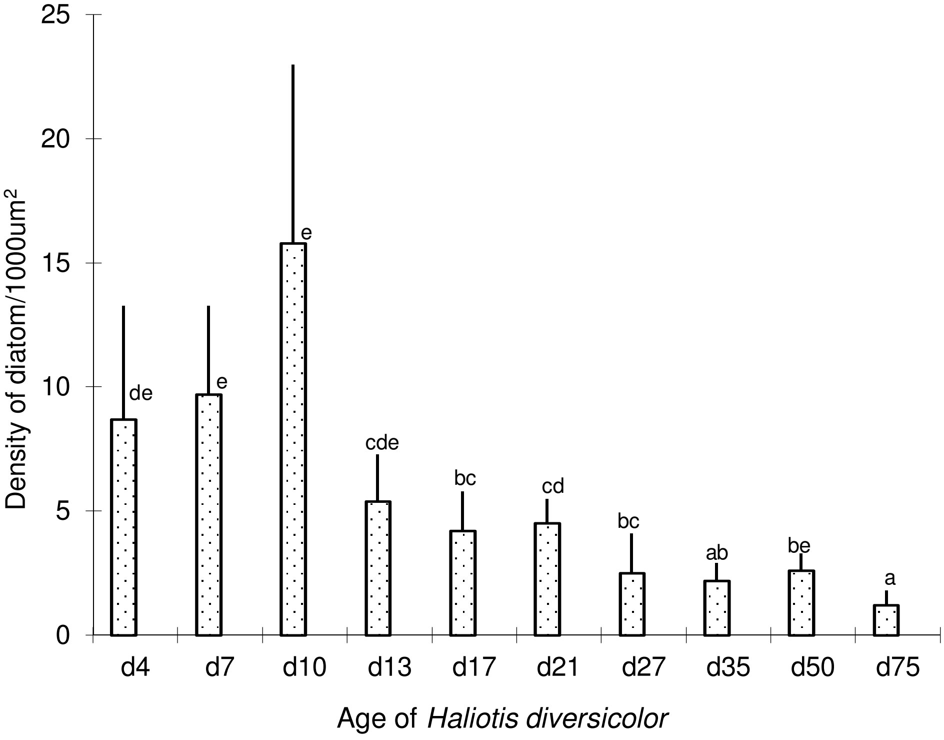

Figure 3. Relative density of ingested diatom in the digestive gut of *H. diversicolor* at different ages (d = day; error bar = SD; common letters denote no significant difference, a<b<c<d<e; n = 10).

DISCUSSION

Previous methods used to describe ingested diatom of abalone were by video recording (Martinez-Ponce and Searcy-Bernal 1998) and analysis of fecal material (Kawamura et al. 1998a). Norman-Boudreau et al. 1986 firstly described the types and kinds of diatoms actively selected by newly-settled abalone and cited by many succeeding authors. Diatoms serve as the main food of postlarval and juvenile tokobushi in the wild and in culture and are important for their growth and survival (Carbajal-Miranda et al. 2005). Diatoms are nutrient-rich because in addition to their cellular contents they have extracellular polymer substances or mucilages, such as the tubes of *Navicula*, pads of *Asterionella*, fibrils of centric diatoms and adhering film of pennate diatoms that act as collecting apparatus for nutrients and microbes (Hoagland et al. 1993). Tokobushi appears to start feeding after settlement since diatoms were present in the stomach of 4 day-old, which was also suggested by Chen (1989). Similar observation was reported in 2 to 4 day-old *H. rufescens* (Martinez-Ponce and Searcy-Bernal 1998) and 2 to 6 day-old *H. midae* (Matthews and Cook 1995).

Most of the diatoms ingested by tokobushi were similar to those given to other abalone during culture. *Cocconeis* dominates the initial prostrate diatom biofilm (Matthews and Cook 1995) and has high digestion efficiencies (Takami 2005). *Nitzschia* and *Navicula* are abundant in a diatom assemblage (Daume and Ryan 2004, Kawamura et al. 2004) and produce high survival (Najmudeen and Victor 2004) and growth (Uki and Kikuchi 1979) to young abalone. *Navicula* and *Melosira* become abundant at the later phase of culture in tanks (Stott et al. 2004), which may partly explain the composition and size of diatom ingested by tokobushi. Other diatoms ingested by tokobushi such as *Thalassionema*, *Triceratium*, *Odontella*, *Licmophora*, *Asterionella* and *Thalassiosira* were not frequently mentioned in abalone literatures probably because they were not cultured or unidentified thus unreported. In general, the growth of postlarval and juvenile abalone is influenced by density, nutritional value and digestibility of diatoms (Kawamura et al. 1998b, Searcy-Bernal et al. 2001, Gorrostieta-Hurtado and Searcy-Bernal 2004, Gordon et al. 2006). These observations were also corroborated by Capinpin (2007).

Data from this study suggest that smaller tokobushi postlarvae could take in small diatoms only but larger ones could have small and large diatoms at the same time. Small diatoms like *Cocconeis* and *Navicula* were consistently ingested by 4 to 75 day-old tokobushi while large or long ones like *Asterionella* and *Thalassiosira* were taken in only by 17 to 75 day-old. The ability of larger tokobushi to ingest larger diatoms appears to be related to increasing size of the digestive system. Roberts (1999) also showed that larger abalone had better developed radula that can be used to detach different types of diatoms. Smaller ones have less developed radula (i.e., highly curved teeth, low clearance angle) that merely function as scoops to collect smaller diatoms. This was corroborated by Onitsuka et al. (2004). The ability of larger tokobushi to ingest larger diatoms appears to be related to the increasing size of the digestive system which coincides with their increasing growth. This may also explain the significantly smaller size and fewer kinds of diatoms ingested at 4 day-old tokobushi compared to 17, 27, 35 and 50 day-old. Similarly, *H. discus hannai* larva of about 500 µm SL could ingest *Cocconeis* with 19 µm cell length and 13 µm cell width (Kawamura and Takami 1995) but *H. iris* postlarva less than 1300 µm SL could not take in *Achnanthes* with 76 µm cell length, 27 µm cell width and long stalks (Kawamura et al. 1998a). The young postlarvae (4 to 10 day-old) may not be able to digest diatoms at this stage may be due to poor digestibility as seen through the fecal material (Kawamura et al. 1998b) and because they lack digestive enzymes to do so (Takami et al. 1998). Hence, they were observed whole and intact in the gut. On the other hand, larger postlarvae are able to break open the frustules and digest the diatoms; that is why maybe few intact diatoms (particularly *Cocconeis*) were observed in the gut.

Other diatom grazers are passive feeders and do not feed based on morphology or size of microalgae but shift from one food to another due to changes in relative amounts or attachments of different diatoms (Aberle et al. 2005). Tokobushi at juvenile stage are capable of grazing on some young macroalgae but they remain feeding on diatoms present or epiphytic to macroalgae. This condition was also observed in other young and even adult abalone (Takami et al. 2003, Simental et al. 2004). The inconsistencies in the number, size and relative density of ingested diatoms by 75 day-old when compared with other younger tokobushi may be related to its tendency to graze on young macroalgae as observed on the plates in tanks, which may result to lesser diatom intake. In *H. discus discus*, the shift in feeding habit from microalgae to macroalgae occurs at about 20 mm SL (Kiyomoto and Yamasaki 1999).

The exponential growth pattern exhibited by 4 to 75 day-old tokobushi is probably due to increasing diatom intake in terms of its composition and size. Similar pattern of growth has been reported in *Haliotis varia* (Najmudeen and Victor 2004). At this stage, metabolic rates are high (Shilling et al. 1996) and there is a greater need for nutrients to support increasing growth rate (Searcy-Bernal et al. 2001, Daume and Ryan 2004). Moreover, most of their energy budget is utilized for somatic growth thus the exponential increase in size (Peck et al. 1987).

The high relative density of diatom ingested by 4 to 10 day-old tokobushi was probably due to small radula, mouth and stomach at this stage (Onitsuka et al. 2004, Roberts et al. 1999). Again, this may be due to the fact that the diatoms were not efficiently digested by young postlarvae at this stage, hence can be seen whole and intact. At this stage, they may even utilize the bacteria, mucus, and diatom extracellular substances as their initial source of energy (see Kawamura et al. 1998b). As mentioned previously, a young postlarval tokobushi has slow growth and just begun feeding on small diatoms able to fit its mouth. It follows therefore that intake maybe maximal relative to size to get enough nutrients for survival and much-needed growth. Ingestion and digestion of diatoms by tokobushi can be affected by diatom size, morphology, attachment and frustule strength (Kawamura et al. 1998a). In this study, the ingested diatom in the stomach of young tokobushi increases in terms of composition and size but inversely proportional to relative density as tokobushi grows bigger. This study shows that histological examination can be one of the methods to characterize the diatom composition, size and relative density of ingested diatoms.

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