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New diets with potential for enhancement of juvenile bivalve seed production and culture techniques

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Abstract: Environmental changes are now having a negative impact on a number of aquatic organisms. In the decades after the mid-1980s, annual catch of bivalves such as the Manila clam (*Ruditapes philippinarum*) continues to decrease drastically in the coastal waters of Japan, and several causative factors have been reported. To conserve the bivalve resources, the promotion of efficiency and stabilization of seed and juvenile bivalve production is needed in addition to the promotion of marine ecosystem and habitat recovery. Under these circumstances, a persistent problem is insufficient food supply for juvenile bivalves because of the difficulty in stable production of diet microalgae at low cost and a deficiency of microalgae that possesses all the necessary dietary requirements. Thus, there is a compelling need for the development of dietary-supplements and/or new species of diet microalgae that contain the essential nutritional properties, and of alternative feeds to replace live microalgal diets. In this mini-review, new diet microalgae, a dietary-supplement, and original feeds using microencapsulation, enzymatic decomposition and fermentation technology with potential for the enhancement of juvenile bivalve seeding production and culture techniques are introduced.

Key words: alginate hydrolysate, *Eutreptiella eupharyngea*, lipid-walled microcapsule, marine silage, seeding production of bivalves

Introduction

Human activities including greenhouse gas emissions have led to increasing global temperatures, perturbed regional weather patterns, rising sea levels, acidifying oceans, changed nutrient loads, and altered ocean circulation (Brierley *et al*., 2009). Such a changing environment is now having a negative impact on a number of aquatic organisms (Brierley *et al*., 2009; Rodolfo-Metalpa *et al*., 2011; Kroeker *et al*., 2013). In particular, there are growing concerns about impacts of environmental change on microalgae, which are the essential primary producers in marine and freshwater aquatic ecosystems. Boyce *et al*. (2010) analyzed the data on available ocean transparency measurements and *in situ* chlorophyll observations to estimate the time dependence of phytoplankton biomass at local, regional and global scales since 1899, and concluded that global phytoplankton concentration has declined over the past century. They also indicated that these fluctuations are strongly correlated with basin-scale climate indices, whereas long-term declining trends are related to increasing sea surface temperatures (Boyce *et al*., 2010).

In Japan, several studies have indicated dramatic decreases in fisheries production and environmental and ecosystem changes in coastal areas such as long-term decreasing trends of phytoplankton biomass and nutrient concentration, long-term changes in species composition of phytoplankton, and increasing sea surface temperatures (Wanishi, 2005; Noda and Yukihira, 2013; Abe, 2017; Yamamoto, 2019; Nishiwaka, 2019; Kaeriyama *et al*., 2019). In the decades after the mid-1980s, annual catch of bivalves, especially the Manila clam (*Ruditapes philippinarum*),

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in coastal waters of Japan continues to decrease drastically. Though the precise cause is unclear, a number of factors affecting the dramatic decrease of the clam species (including overfishing) have been suggested (Hamaguchi *et al.*, 2002; Paillard, 2004; Park *et al.*, 2006; Tsutsumi, 2006; Matsukawa *et al.*, 2008; Toba *et al.*, 2013; Toba, 2017). Furthermore, a wide variety of studies have aimed at conserving the clam resource (Dang *et al.*, 2010; Paul-Pont *et al.*, 2010; Shigeta and Usuki, 2012; Suzuki *et al.*, 2012; Usuki *et al.*, 2012; Kobayashi *et al.*, 2012; Ikushima *et al.*, 2012; Sakurai *et al.*, 2012; Hasegawa *et al.*, 2012; Nakagawa *et al.*, 2012; Sakami and Higano, 2012; Houki *et al.*, 2015; Hanyu *et al.*, 2017; Hata *et al.*, 2017; Houki *et al.*, 2018). To conserve the clam resource, however, promotion of efficiency and stabilization of seed production and juvenile clam culture are needed.

In this mini-review, new diet microalgae, a dietary-supplement, and original feeds using microencapsulation, enzymatic decomposition, and fermentation technology with potential for the enhancement of juvenile bivalve seeding production and culture techniques are reviewed.

Dietary Effect of a New Diet Microalgae and Mixed Algal Diets on Juvenile Bivalves

Microalgae are used as live feeds for larval or juvenile bivalves, crustaceans and other invertebrates in addition to the rotifer *Brachionus plicatilis*, which is fed to larval fish. Thus, diet microalgae should contain essential nutritive constituents, be nontoxic, be appropriately sized for ingestion, and possess digestible cell walls to allow the nutrients to be absorbed after ingestion (Becker, 2004). In addition, it is important that the microalgae can be produced in large quantities at low cost. So far, more than 40 species of microalgae have been isolated and analyzed to produce better aquaculture feeds, and these species are undergoing cultivation as pure strains in intensive systems (Becker, 2004). However, there is no microalgae that possesses all the necessary requirements of an ideal diet alga. Therefore, the development of such new diet microalgae could be crucially important for the enhancement of seed production and culture of juvenile bivalves.

As described in the Introduction, a variety of studies have sought to conserve wild *R. philippinarum* populations while providing a stable market supply through developing clam culture. A persistent problem, however, is insufficient food supply for juvenile clams exceeding a shell length of 1 mm (especially in late fall and spring) since clams have high food requirements, and low temperature slows the growth of the diet microalgae. Therefore, the development of new species of diet microalgae that can grow well outdoors at low water temperatures and that possess the essential nutritive constituents will greatly benefit juvenile clam culture. Recently, Yamasaki *et al.* (2019) isolated the marine euglenophyte *Eutreptiella eupharyngea* from a pond used for extensive phytoplankton cultivation at the Yamaguchi Prefectural Fisheries Research Center (Yamaguchi, Japan) in January 2013. The study reported from the results of both laboratory and outdoor experiments that *E. eupharyngea* could grow well at water temperatures ranging from 4 to 25℃, but could not grow at 30℃. Furthermore, Yamasaki *et al.* (2019) demonstrated that the dietary effect of *E. eupharyngea* per dry weight, on juvenile *R. philippinarum* of more than 1.5 mm shell length, exceeded that of the diatom *Chaetoceros neogracile*—a known suitable diet alga for juvenile clams. These findings were attributed to the high nutritional value of *E. eupharyngea* as typified by its high protein and sugar content and high content ratio of n-3 fatty acids such as eicosapentaenoic and docosahexaenoic acid and n-6 fatty acids such as arachidonic acid. In addition, *E. eupharyngea* (**Fig. 1**) appears to have a stronger dietary effect on bigger clams (e.g., more than 1.5 mm in shell length) since *E. eupharyngea* cells (cell length: 35–70 μm; cell width: 7.5–12 μm, Walne *et al.*, 1986) are bigger than other diet microalgae such as *C. neogracile* (cell size: <10 μm). Thus, *E. eupharyngea* shows considerable potential to become a new diet alga for the seed production and culture of juvenile bivalves in late fall and spring.

A multitude of studies suggest that mixed microalgal diets may provide a better balance of essential nutrients to bivalves. Rivero-Rodríguez *et al.* (2007) analyzed the relative fatty acid

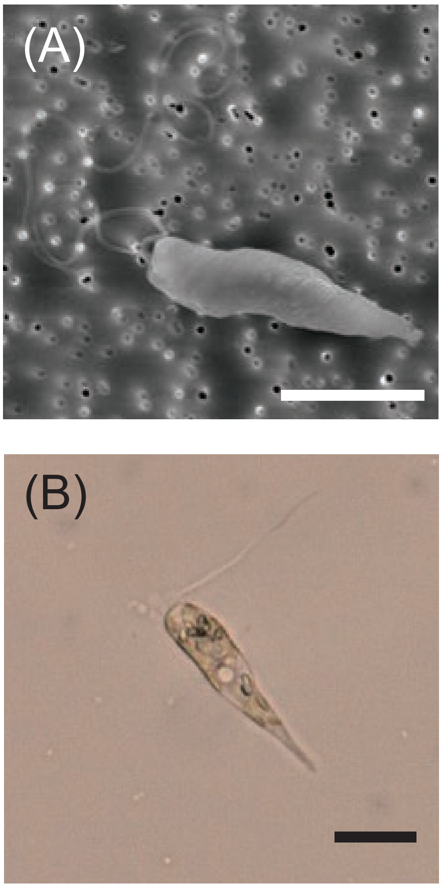

Fig. 1. A scanning electron microscope (SEM) photograph (A) and a micrograph (B) of *Eutreptiella eupharyngea*. The scale bars indicate 10 µm.

composition of the diatoms *Chaetoceros calcitrans, C. muelleri,* and *Phaeodactylum tricornutum*, as well as the haptophyte *Isochrysis galbana* and the prasinophyte *Tetraselmis suecica*, and reported that these microalgae contained a high proportion of either EPA (C20 : 5) or DHA (C22 : 6). Furthermore, they examined the dietary contribution of these five algal species when provided as mono- or bi-algal diets to juveniles of the mangrove oyster (*Crassostrea corteziensis*), and concluded that *C. calcitrans* provided the best diet, probably due to its high AA (C20 : 4) content (Rivero-Rodríguez *et al*., 2007). In addition, Ronquillo *et al.* (2012) examined the effect of mixed microalgal diets on the growth and fatty acid profile of European flat oyster (*Ostrea edulis*) juveniles. The authors indicated that dietary effect of the mixture of the eustigmatophyte *Nannochloropsis oculata* and the haptophyte *Pavlova lutheri*, which have had higher levels of polyunsaturated fatty acids (PUFAs), was higher than that of other combinations of diet microalgae. However, Geng *et al.* (2016) examined the effects of four different microalgae, *C. calcitrans*, *I. galbana*, *N. oculata* and *Diacronema viridis*, on the growth of juvenile ark shells (*Tegillarca granosa* Linnaeus), and demonstrated that the best feeding effects were observed with the mixture of all four microalgae, and binary algal diets were second best. Recently, Liu *et al.* (2016) evaluated the nutritional value of eight species of microalgae for larvae and early post-set juveniles of the Pacific geoduck Clam (*Panopea generosa*), and reported that a balanced mixture of various dietary nutrients was important. In particular, they suggested that the ratios between n-3 and n-6 fatty acids, and between EPA and DHA, are especially crucial (Liu *et al*., 2016). Polyunsaturated fatty acids (PUFAs), especially the n-3 fatty acids eicosapentaenoic acid (EPA, C20 : 5n-3) and docosahexaenoic acid (DHA, C22 : 6n-3) play an important role in bivalve growth and development (Volkman amd Brown, 2005; Martínez-Fernández *et al*., 2006). Thus, the fatty acid composition of diet microalgae might be one of the most important factors for bivalve growth and development.

Nevertheless, content and/or composition of these fatty acids could be affected by microalgal growth rates, environmental conditions, and growth phases (Richmond, 1986). The main environmental factors affecting the growth and the nutritive constituents of diet microalgae are light levels, nutrients, temperature, pH, and salinity (Chu *et al*., 1996; Tzovenis *et al*., 1997; Zhu *et al*., 1997). The impact of increasing water temperature and ocean acidification should also be considered.

Enhancement of Dietary Effect on Juvenile Bivalves by the Dietary Supplements

Jǿrgensen (1983) observed that clams take up dissolved organic matter (DOM) in seawater through epidermal tissue in the mantle and gills. Welborn and Manahan (1990) showed that larvae of the bivalve *Crassostrea gigas* (Pacific oyster) can take up dissolved glucose, maltose, cellobiose, and cellotriose, but not rhamnose or maltotriose. In addition, Uchida *et al.* (2010) reported that the growth rate of soft tissue in *R. philippinarum* was significantly promoted by supplementing a diet of the diatom *C. calcitrans* with glucose at concentrations of 10 and 100 mg L^{-1}. Furthermore, Taga *et al.* (2013) reported the high dietary effects of the raphidophyte *Heterosigma akashiwo* (known as a harmful algal species) on juvenile *R. philippinarum* and suggested that the acidic sugars found in microalgal cells may be one of the important factors determining the growth of juvenile clams. Thus, we focused on alginate—a known acidic sugar.

Alginate is a natural acidic linear polysaccharide that is composed of α-L-guluronate and β-D-mannuronate (uronic acids) residues. This carbohydrate occurs in the cell walls of brown algae, such as *Saccharina japonica* and *Undaria pinnatifida*. Alginate and its hydrolysates are currently used in a wide range of commercial products because of their safety, low price, and bioactivities. Since alginate is poorly soluble in water, we focused on the dietary effect of alginate hydrolysates which are water soluble and thus usable by clams. Results showed that shell-length growth of juvenile *R. philippinarum* was significantly promoted by supplementing a diet of *C. neogracile* with alginate-hydrolysates (AHs) of at least 1 mg L^{-1}. The most effective concentrations of AHs were 2 to 4 mg L^{-1}, but shell length growth in groups given AHs only without *C. neogracile* was significantly inhibited (Yamasaki *et al*., 2015). In addition, Yamasaki *et al.* (2016) demonstrated that growth of adult clams (initial average shell length [±SD], 15.7±0.3 mm) was dramatically promoted by supplementing a diet of the diatom *C. neogracile* with AHs at 4 mg L^{-1}, and metabolomics indicated that each of the states of starvation, food satiation, and sexual maturation of *R. philippinarum* has a characteristic pattern in the metabolite profile (Yamasaki *et al*., 2016).

A few kinds of *Chaetoceros* spp. are widely used for clam culture, but the cost of cultivating *Chaetoceros* spp. is expensive. *Nannochloropsis* spp. are used for cultivation of a various marine molluscs, crustaceans, and zooplankton and can be produced in large quantities at low cost (Zhang *et al.*, 2001). However, the dietary effect of *Nannochloropsis* spp. on juvenile bivalves is not always sufficient as compared to other diet microalgae. Accordingly, we tried to enhance the dietary effect of *Nannochloropsis* sp. on the shell length growth of juvenile *R. philippinarum* by AHs supplementation. Yamasaki *et al.* (2018) reported that shell length and total weight of clams were significantly promoted by supplementing a diet of *Nannochloropsis* sp. at the concentration of 30×10^4 cells mL^{-1} with AHs at the concentration of 4 mg L^{-1} as compared with the groups given *Nannochloropsis* sp. Thus, a combination of *Nannochloropsis* sp. and AHs will be useful to shorten the rearing period of clams at low cost since shell length growth of the clams fed *Nannochloropsis* spp. added with AHs were faster than that of clams fed more costly *Chaetoceros* spp.

Particulate organic matter (POM) appears to contribute to improvements in the quality of bivalves. To improve the quality of the freshwater Clam (*Corbicula japonica*) by rearing in a short period, Nojiri *et al.* (2018) examined the effect of various carbohydrates on the increment of glycogen content, and of the hyperosmotic stress on the amino-acid uptake. As a result, Nojiri *et al.* (2018) showed that rice powder was effective for increasing glycogen content at the concentration of 0.1g/L, and suggested that insoluble carbohydrate was suitable for the increment of glycogen content in the freshwater clam. Furthermore, they observed that glycine was most effectively absorbed in the freshwater clam, followed by proline, alanine and glutamic acid under hyperosmotic stress for 24 h, and concluded that the quality of *C. japonica* could be improved in a short period by feeding rice powder and rearing in palatable amino acids under osmotic stress.

New Approaches in the Development of Original Feeds for Bivalves

There is a compelling need for the development of alternative feeds to replace live microalgal diet because of the difficulty in stable production of diet microalgae at low cost and a deficiency of microalgae that possesses all the necessary requirements for a diet alga. In this section, several new approaches in the development of original feeds using microencapsulation, enzymatic decomposition and fermentation technology for bivalves are introduced.

Recently, several studies have suggested that microcapsules, which can easily be produced in large quantities, have highly customizable physical characteristics and contents, and are stable for long term storage (Aldridge *et al*., 2006; Costa *et al*., 2011). Thus, microcapsules have promise as alternative feeds to replace a live microalgal diet. Willer and Aldridge (2017) demonstrated that a new form of microencapsulated diet known as BioBullets (BioBullets Ltd., Cambridge, UK) can successfully be ingested by the blue mussel (*Mytilus edulis*). Furthermore, Willer and Aldridge (2019) demonstrated that the use of microencapsulated feed, which were lipid-walled microcapsules containing 50% powdered *Schizochytrium* algae by weight and manufactured by BioBullets (BioBullets Ltd.), can lead to major improvements in survivorship and growth in juvenile European flat oysters (*Ostrea edulis*).

Several studies have also suggested the viability of marine silage (MS) and single cell detritus (SCD; Uchida and Murata, 2002; Pérez Camacho *et al*., 2004; Uchida *et al*., 2004). MS and SCD can easily be produced in large quantities, have a suitable size for ingestion by filter feeder such as bivalves, and are stable for long term storage without remarkable loss of particulate products. Thus, MS and SCD have promise as alternative feeds to replace live microalgal diet. Pérez Camacho *et al.* (2004) showed that SCD prepared from *L. saccharina* using enzymatic and bacterial decomposing activities has some dietary effect on the clam *Ruditapes decussatus*. Though shell length growth of the clam spats fed *C. gracilis* was higher than those fed PS, Kalla *et al.* (2008) reported that spheroplasts prepared from *Porphyra yezoensis* (Rhodophyta) using enzymatic decomposition had some dietary effect on the *R. philippinarum* spat. In addition, Uchida and Murata (2002) suggested "marine silage" prepared from *U. pinnatifida* as a novel fisheries-diet, which is produced from the combination of conversion of seaweed to SCD and induction of lactic acid fermentation utilizing activities of a lactic acid bacterium and yeast. Furthermore, Uchida *et al.* (2004) demonstrated that MS prepared from *U. pinnatifida* had a limited but positive dietary effect on Japanese pearl oyster (*Pinctada fucata martensii*) spat.

Conclusion

Research and development of new diets such as diet microalgae, dietary supplements, and original feeds using microencapsulation, enzymatic decomposition and fermentation technology are rapidly developing. In the near future, efficiency and stabilization of seed production and juvenile clam culture may be achieved by a combination of new diets and live algal diets. Therefore, these techniques may have important implications for clam culture, and could contribute to the conservation of the wild clam resources and a stable market supply. Further studies are needed to develop a process for practical utilization of these techniques for seed production and juvenile clam culture.

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Annotated Bibliography of Key Works

(1) Yamasaki Y., Taga S., and Kishioka M., 2015: Preliminary observation of growth-promoting effects of alginate hydrolysates on juvenile Manila clams, *Ruditapes philippinarum*. *Aquaculture Res.*, **46**, 1013–1017.

Several studies have suggested that certain types of sugars are potentially a good supplement for growth of bivalves such as *Ruditapes philippinarum*. We observed the dietary effects of a harmful raphidophyte *Heterosigma akashiwo* on juvenile clams and suggested that the acidic sugars in the phytoplankton might be an important factor determining the shell length growth of clams because total sugar and acidic sugar content of *H. akashiwo* were higher than other diet microalgae. Therefore, we focused on alginate known as one of the acidic polysaccharides, and showed that shell-length growth of juvenile clams (average shell length: 432 to 507 μm) was significantly promoted by supplementing the diatom *Chaetoceros neogracile* (40,000 to 80,000 cells mL^{-1}) with alginate-hydrolysates (AHs) of at least the concentration of 1 mg/L. In addition, the most effective concentrations of AHs were 2 to 4 mg L^{-1}.

(2) Yamasaki Y., Taga S., Kishioka M., and Kawano S., 2016: A metabolic profile in *Ruditapes philippinarum* associated with growth-promoting effects of alginate hydrolysates. *Sci. Rep.*, **6**, 29923.

We demonstrated that shell length growth of *Ruditapes philippinarum* (average shell length: 15.7 mm) was significantly promoted by supplementing the diatom *Chaetoceros neogracile* (80,000 cells mL^{-1}) with alginate-hydrolysates (AHs) at the concentration of 4 mg L^{-1}. Furthermore, metabolomics indicated that clams in the groups given *C. neogracile* with AHs at the concentration of 4 mg L^{-1} actively utilized excess carbohydrate for the development of reproductive tissue. On the other hand, clams in the groups given *C. neogracile* only were actively growing through the use of their adequate carbohydrate resources. Thus, supplementation of AHs with the algal diet may be an effective way to shorten the rearing period of clams.

(3) Yamasaki Y., Ishii K., Taga S., and Kishioka M., 2018: Enhancement of dietary effect of *Nannochloropsis* sp. on juvenile *Ruditapes philippinarum* clams by alginate hydrolysates. *Aquaculture Rep.* **9**, 31–36.

The eustigmatophyte *Nannochloropsis* sp. is widely used in the aquaculture industry because this species can be produced on a large scale at low cost. However, *Nannochloropsis* sp. has less dietary effect on juvenile bivalves compared with other diet algae such as the diatom *Chaetoceros neogracile* and the haptophyte *Diacronema* (=*Pavlova*) *lutheri*. In this study, the use of alginate-hydrolysates (AHs) to enhance the dietary effect of *Nannochloropsis* sp. on juvenile *Ruditapes philippinarum* (average shell length: 1,090 μm) was attempted. As a result, enhancement of the dietary effect on shell-length growth of juvenile clams was observed in the groups given *Nannochloropsis* sp. (300,000 cells mL^{-1}) with AHs at the concentration of 4 mg L^{-1}. Hence, the enhanced dietary effect of a combination of *Nannochloropsis* sp. and AHs will be useful to shorten the rearing period of *R. philippinarum*.

(4) Yamasaki Y., Ishii K., Hikihara R., Ishimaru M., Sato F., Taga S., Kishioka M., Matsunaga S., Shikata T., Abe M., Kato S., Tanaka R., and Murase N., 2019: Usefulness of the euglenophyte *Eutreptiella eupharyngea* as a new diet alga for clam culture. *Algal Res.*, **40**, 101493.

Microalgae are an essential feed source for seed production of bivalves such as *Ruditapes philippinarum*. However, there is a deficiency of microalgae that can provide a stable supply of nutrient-rich feed at low water temperatures during winter and spring. To develop a new diet of microalga that can grow well outdoors at low water temperatures and possesses the essential nutritive constituents, we focused on the euglenophyte *Eutreptiella eupharyngea*, which was isolated from a pond used for extensive phytoplankton cultivation at the Yamaguchi Prefectural Fisheries Research Center (Yamaguchi, Japan) in January 2013. As a result, this species grew well at water temperatures of 10–25℃, but could not grow at 30℃. Furthermore, the dietary effect of *E. eupharyngea* per dry weight on juvenile *R. philippinarum* (average shell length: 1,426 μm) exceeded that of the diatom *Chaetoceros neogracile*. These findings are attributable to the high nutritional value of *E. eupharyngea* as typified by its high protein and sugar content and high content ratio of n-3 fatty acids such as eicosapentaenoic and docosahexaenoic acid and n-6 fatty acids such as arachidonic acid.