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# Methane-Oxidizing Bacteria as Feed Replacement for Blue Mussel (*Mytilus edulis*) Larviculture

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**Key words:** Methane-oxidizing bacteria, Mussel larvae (*Metylus edulis*), Diluted ammonium mineral salt, Nitrate mineral salt, Larviculture

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## Abstract

Bacterivory is common in bivalves. To test the effect of methane-oxidizing bacteria (MOB) on the growth and survival of blue mussel (*Mytilus edulis*) larvae, MOB was used as a replacement to microalgae. Enriched and sub-cultured MOB is from the marine sediment sample from the Northsea coast of Yeserke, The Netherland. The feeding ratio of MOB was 75% 50%, and 25% combined with 25%, 50% and 75% microalgae, respectively, based on dry weight. Control treatments are 50% and 100% microalgae only. The microalgal diet used consisted of a combination of *Isosychris galbana*, *Chaetoceros muelleri* and *Tetraselmis* suecica. Growth and survival of mussel larvae fed with MOB showed no significant difference compared with the 100% microalgae diets on day 6. Average shell height among all treatments was 80.7 ± 1.99 µm and an average survival rate of 11.12 ± 2.61%. Nevertheless, due to massive mortality due to the *Vibrio sp*. attack in the laboratory the experiment was terminated on day 8. This study, however, even at a short time still displayed that the use of MOB offers a promising result as a replacement of live algae for mussel larvae. The data of this study provide good insights regarding MOB as a possible bacterial meal for mussel larviculture. More future research is needed on the application of the MOB as feed for mussels since this is the first time that the MOB is applied as food for blue mussel larvae.

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**Introduction**

Large-scale production of an aquatic organism is a challenging task; especially the early life stages are very vulnerable. The quantity and quality of production of aquaculture farming are mainly affected by the quality of the seed (SEAFDEC, 2014). However, "larviculture" techniques differ between species. But a crucial aspect of larviculture, in general, is the quality of feed given to the animals and to what extent it matches the nutritional requirements in these initial feeding phases.

Bivalve larvae filter-feed as soon as they reach the D-larval stage, 48 hours after fertilization, on single cells such as bacteria and algal picoplankton (Doroudi *et al.* 1999, Tomaru *et al.* 2000). Bivalve hatcheries usually feed the larvae with a mixture of different strains of microalgae as their carbon and nitrogen sources. The typically used microalgae are *Chaetoceros calcitrans*, *C. muelleri*, *Isosychris spp*, *Tetraselmis spp, etc* (Brown, 2002). Nevertheless, the production of microalgae poses several shortcomings such as the high production cost, labor-intensive (Coutteau and Sorgeloos, 1992), risk of contamination and temporal variation in the nutritional value (Pauw *et al*., 1984). Thus, due to these algae production remains a major bottleneck for the commercial production of seed in bivalve hatcheries.

This can be resolved, however, by replacing the fresh microalgae with dried algae, algae concentrate, or yeast-based diets (Coutteau and Sorgeloos, 1992; Aji, 2011) and even single-cell protein (SCP) (Ho *et al*., 2013), the latter is having a much cheaper production cost among the options.

To show other perspectives of feeding, some ubiquitous bacteria are good candidates for microalgae replacement. Bacteria are, nowadays, widely known and promising cheap sources of carbon and nitrogen for aquaculture-raised organisms such as methane-oxidizing bacteria (MOB) that are found ubiquitously such as in the water column, sediment, or water-sediment interface and that are easily enriched and isolated (Leak and Dalton, 1986; Ha *et al*., 2012). Methane oxidizing bacteria, though not all, also produce poly-hydroxybutyrate (PHB) (Leak, 1992) that can act as an immune-stimulant (Da Muller, 2006), the essential amino acid methionine, vitamins such as cytochrome *c* (Leak, 1992) and B12 (Ivanova *et al*., 2006) and coenzymes (Leak, 1992). MOB also has a high concentration of K, Mg, and Fe (Ku´zniar *et al*., 2019).

The main factor that made methanotrophs renowned in the biotechnology industry is the comparatively low cost of methane as carbon substrates for production. Currently, methanotrophs are used as a source of the bacterial meal (BM), a protein source or fodder biomass for aquaculture farmed organisms (БабусенкоВалерий, 2020). *Methylococcus capsalatus* (Bath), was also proven that it is a promising source of protein since it has amino acids that can support the need of some of the monogastric animals such as Atlantic salmon (*Salmo salar*), rainbow trout (*Onchorynchus mykiss*) and even Atlantic halibut (*Hippoglossus hippoglossus*). It is also digestible and offers good animal performance and health (Overland, *et al*., 2009). These features and the fact that they measure on the average size of 0.6 to 5 μm (Heyer *et al*, 2002; Losekann *et al*, 2007) make them good candidates for use as live food for bivalve larvae.

The objective of this research is to know the effect of MOB as a partial feed replacement to live microalgae without negatively affecting the growth and survival of mussel larvae.

**Materials and methods**

*Production of protein cells for mussel larvae feeding*

*MOB production*

*Enrichment and Subculture of Methane Oxidizing Bacteria*

The production flow of the MOB as shown above (Fig. 1) started from the enrichment stage to the large-scale production of the selected medium for MOB used for food replacement. 10-gram sediment from the 5-10 cm deep of the Northsea coast of Yeserke, The

Netherlands was used as inoculum. Samples were placed in a 1L Schott® bottle supplemented with 200 ml of different culture medium (Table 1) for enrichment namely the ammonium mineral salt medium (AMS) and its diluted form (dAMS), and nitrate mineral salt (NMS) and its diluted form (dNMS). A 200 ml (% v/v) of methane gas was added to all bottles as the sole carbon source in stimulating the growth MOB. This value was based on Whittenbury, 1976. Each treatment was triplicated and was stored for five weeks in a dark room on a shaker at 28°C.

Monitoring and analysis of methane, carbon dioxide and oxygen gases in the headspace of each bottle were done daily using the Global Analyser Solution ® compact gas chromatographer.

As the enrichment samples reached 0.2 to 0.8 optical density at 610 nm ($OD_{610}$), a subsequent subculture was done per medium and triplicated. Twenty milliliter of inoculum from the enriched samples was taken from each bottle and added to a new 1L Schott® bottle with 180 ml of each salt medium and 200 ml (% v/v) of methane gas. This was observed for 6 days in the same storage room condition.

The subculture that showed the highest $OD_{610}$ value, based on the measured turbidity (Bussman, *et al*., 2004), which was the dAMS, was selected for the up-scale production of MOB protein cells that were used for the feeding replacement of the mussel larvae. For upscaling, the large bottles used were 1L, 2 L, 5 L and 10 L bottles for production and methane gas in the headspace (20% of the total fluid volume of the bottle) was monitored and analyzed every 2 days using a gas chromatographer.

*Flow cytometry reading*

The samples used for turbidity reading were further analyzed by flow cytometry to determine cell concentration. The samples were diluted 100x, 1000x, and 10,000x if the $OD_{610}$ reading with spectrophotometer were between 0.06 - 0.09, 0.1 -1.5 and 1,5 to 2.5, respectively.

The live and dead cell ratio was estimated using the combined solution (SG/PI) of the Sybe Green stain (SG), which stains the total cells and propidium iodide (PI), which stains the damage or dead cell. As such, the ratio between dead and live cells can be determined. The diluted samples in a plastic tube (500 µL) were stained with 5 µL of SG/PI and were mixed using a vortex (Ruger *et al*., 2014). The stained samples were incubated at 37°C for 13 minutes and further analyzed with the BD Accuri® C6 Flow cytometer.

*Harvesting of MOB*

For a continuous production and harvesting of MOB protein cells, the large scale production of MOB was done by batch every after 2 days. The turbidity of the MOB culture was checked visually. The more turbid batch cultures were selected for the harvest. There were 7 liters in a total of MOB that was centrifuged using Thermo Fisher Scientific® at 10,000 rpm for 10 minutes in 50 mL falcon tubes. Further, the supernatants were discarded and the pellets were pipetted from the falcon tubes and pooled in a 2 L Schott® bottle with 2 L distilled fresh water and stored in 4°C. The optical density and the cell concentration were checked every before feeding the mussel larvae.

*Algae production*

There were 3 species of microalgae used in the study namely *Isosychris galbana, Chaetoceros muelleri* and *Tetraselmis suecica*. The stock culture of algae was grown in 20 mL of autoclaved culture medium for 2 weeks in test tubes. After two weeks the algae was up-scaled to 200 mL and allowed to grow for a week before transferring to a 2 L of culture. After growing in 2 L for 3 to 4 days, the algae were transferred to a 20 L Schott® bottle. For the latter volume, the seawater was UV-treated and filter-sterilized to 0.2 µm. The algae were aerated and batch-grown under a 24 hours light regime at 21°C.

*Mussel spawning*

Mussel (*Metylus edulis)* broodstock used was collected from the hatchery of Roem van Yeserke, The

Netherlands. They were washed and stored with a moist cover at 4°C storage. To induce spawning, the mussel broodstock was subjected to thermal shocks. The broodstock was immersed alternatingly in filtered (0.2 µm) natural seawater (FSW) from 24°C to14°C for 30 to 45 minutes time interval. They were, from time to time, given a small number of mixed algae as another spawning stimulant.

When the mussel started to release gametes, the animal was transferred to a separate small container with seawater for eggs or sperm collection. Eggs were not allowed to stay longer than 10 minutes without mixing it with sperm to guarantee good fertilization. Sperms and eggs were mixed and allowed to stay for 15 to 20 minutes to assure fertilization (Beiras and His, 1994). Using a 60 µm sieve, dirt and other debris were separated from the fertilized eggs while the fertilized eggs were collected using a 30 µm sieve; and washed gently and thoroughly to remove the excess of sperm and avoid polyploidy. The rinsed fertilized eggs were then stored for 48 hours under room temperature of 18°C (Rico-villa *et al.*, 2006) in autoclaved seawater in a basin until they reached the D-larvae stage.

*Experimental Set-up*

There were fifteen 10L Zuger bottles (Fig. 2) for five treatments to accommodate the batch culture of larvae. The larvae were stocked at 10 larvae per mL in a total volume of 5L fresh seawater (FSW) with aeration provided with 18 to 19°C room temperature. Water management with a total water change was done every other day, which also coincided with the supplementation schedule of the experimental diets.

*The feeding experiment*

There were five treatments with 5 replicates each. Treatment 1 corresponded with a diet of 50% algae and served as negative control 1. Treatment 2 (75% MOB) based on the dry weight of algae, Treatment 3 (50% MOB), Treatment 4 (25% MOB) and Treatment 5 with a diet of 100% algae as control 2. The microalgae used were isolated and cultured by the researcher itself in the ARC Laboratory.

The amount of MOB given was based on the dry weight of algae which is $20\times10^{-2}$, $70\times10^{-12}$ and $200\times10^{-12}$ g for *Isosychris spp*, *C. muelleri* and *Tetraselmis spp*, respectively. The dry weight of MOB was also assumed as 1 picogram based also on the general standard dry weight of bacteria (Fitzpatrick, J., 2015). The feeding experiment was done every other day good for 20 days of culture (Doroudi *et al.*1999), with a feeding scheme shown in Table 2. However, in this experiment, it only took 6 days of culture due to *Vibrio sp*. attack on the stocks.

*Sampling Method for Growth and Survival of Larvae*

Measurement of the shell height was done by measuring the distance between the umbo and ventral valve margin, using the microscope with a micrometer ruler under 20x magnification. This was done every 4 days to see the difference of growth in between the feeding days. The survival was determined every 2 days after the initial measurement during the first day. The larvae were collected on a 60 µm sieve and concentrated in a beaker. Three sub-replicate of 500 µL of sample for each tank were taken for the survival determination and 50 larvae were used for shell height measurement for each replicate of every treatment.

*Statistical analysis*

Data of survival rate and growth of mussel larvae test of homogeneity of variance and Quantile-Quantile plot was done using SPSS 22. The assumption was met, so the parametric test one-way analysis of variance was used (ANOVA) test at the 0.05 probability level.

**Results**

*Effect of MOB on Growth and Survival of Mussel Larvae*

The effect of MOB on the growth of mussel larvae is represented in Fig. 3. On day 4, there is no significant difference in growth between all treatments which had almost the same average shell heights ranging from 77.9 to 79.2 µm. There is also no significant difference in growth among all treatment ($p > 0.05$)

on day 6, however, with a shell height varying between 84.2 ± 0.37 µm and 79.1 ± 0.08 µm. There were no significant differences (p=0.158) observed for all treatments with MOB replacement compared to 50 and 100% algae (Fig. 4). All treatment dropped to 8.06 to 13.88% survival on day 6 after which it was decided to stop the experiment on day 8. The sampling was done only until day 6 instead of day 20, due to the massive mortality of larvae to all treatments.

**Table 1.** Medium composition and preparation of 1L stock soltuion for MOB (NMS, AMS, dNMS, dAMS) and dAMS) (1L).

| NMS and AMS | dNMS and dAMS |
|---|---|
| 100 mL of salt stock | 20 mL of salt stock |
| 1 mL FeNaEDTA stock | 1 mL FeNaEDTA stock |
| 10 mL Na2HPO4.12H2O stock | 10 mL Na2HPO4.12H2O stock |
| 1 mL Trace solution | 1 mL Trace solution |
| 888 mL distilled water | 968 mL distilled water |

Components: *NMS and AMS salt stock*: $MgSO_4.7H_2O$ (10g/L), $KNO_3$ (10 g/L for NMS), $NH_4Cl$ (5 g/L for AMS), $CaCl_2.2H_2O$ (1.5 g/L); FeNaEDTA (0.5 g/100 ml); $Na_2HPO_4.12H_2O$ (71.7 g/L); trace solution: $Na_2EDTA.2H_2O$ (0.5 g/L), $FeSO_4.7H_2O$ (0.2 g/L), $H_3BO_3$ (0.03 g/L), $CoCl_2.6H_2O$ (0.02 g/L), $ZnSO_4.7H_2O$ (0.01 g/L), $MnCl_2.4H_2O$ (0.003 g/L), $NaMoO_4.2H_2O$ (0.003 g/L), $NiCl_2.6H_2O$ (0.002 g/L); $CuSO_4$ (2.5 g/L for pMMO) and 0.025 g/L to encourage growth sMMO-possessing organisms.

**Table 2.** Feeding regime of mussel larvae based on dry weight (mg) of algae.

| | Day 1,2 | | Day 3,4 | | Day 5,6 | |
|---|---|---|---|---|---|---|
| Treatment | Algae | MOB | Algae | MOB | Algae | MOB |
| 50% algae | 0,15 | 0 | 3,6 | 0 | 4,5 | 0 |
| 75% MOB | 0,075 | 0,225 | 1,8 | 5,4 | 2,25 | 6,75 |
| 50% MOB | 0,15 | 0,15 | 3,6 | 3,6 | 4,5 | 4,5 |
| 25% MOB | 0,225 | 0,075 | 5,4 | 1,8 | 6,75 | 2,25 |
| 100% algae | 0,3 | 0 | 7,2 | 0 | 9 | 0 |

**50% and 100% algae are the control. For algae, based on 100% dry weight of proportion given for 5L: Day 1,2: only *Isosychris spp.*, 30 cells/µL; Day 3,4: for both *Iso* sp.& *Chaetoceros muelleri,* 20 cells/µL ; Day 5,6: *Iso.* & *C. muelleri* 25 cells/µL.

**Discussion**

*Effect of MOB on Growth and Survival of Mussel Larvae*

Co-feeding of algae and bacteria had been described as affecting N and C assimilation in the feeding organisms (Arapov *et al.*, 2010; Toi *et al.*, 2013). Based on this experiment, partial replacement of feeding a mixed culture of MOB had no significant difference in the effect on growth and survival of mussel larvae compared to mussel larvae fed with algae. Nevertheless, this also implies that the replacement of MOB even in 25%, 50% and 75% have the same growth performance compared to 100% algae. This result only gives a promising result of MOB as a replacement for microalgae. It will also lower the demand for microalgae for feeding, which also entails a lower production cost of microalgae since there is a lower demand for use of microalgae for feeding mussel larvae.

However, the response of mussel larvae fed with MOB and algae in this study does not agree with the result of Gatenby *et al.* (1997) and Subhash and Liption (2007) where the mussel larvae had better survival

and growth if fed with algae and bacteria, but not MOB, compared to the solely algae-fed mussel larvae. Moreover, using other species of bacteria, Douillet and Langdon (1993) observed that the larvae had better survival and growth under a co-feeding regime with algae under axenic conditions. Because, as far as we know, there are no related studies of mussel larvae fed specifically with MOB, the result of this live food experiment cannot be fully compared since this is the first time that the MOB is used as live food replacement for microalgae.

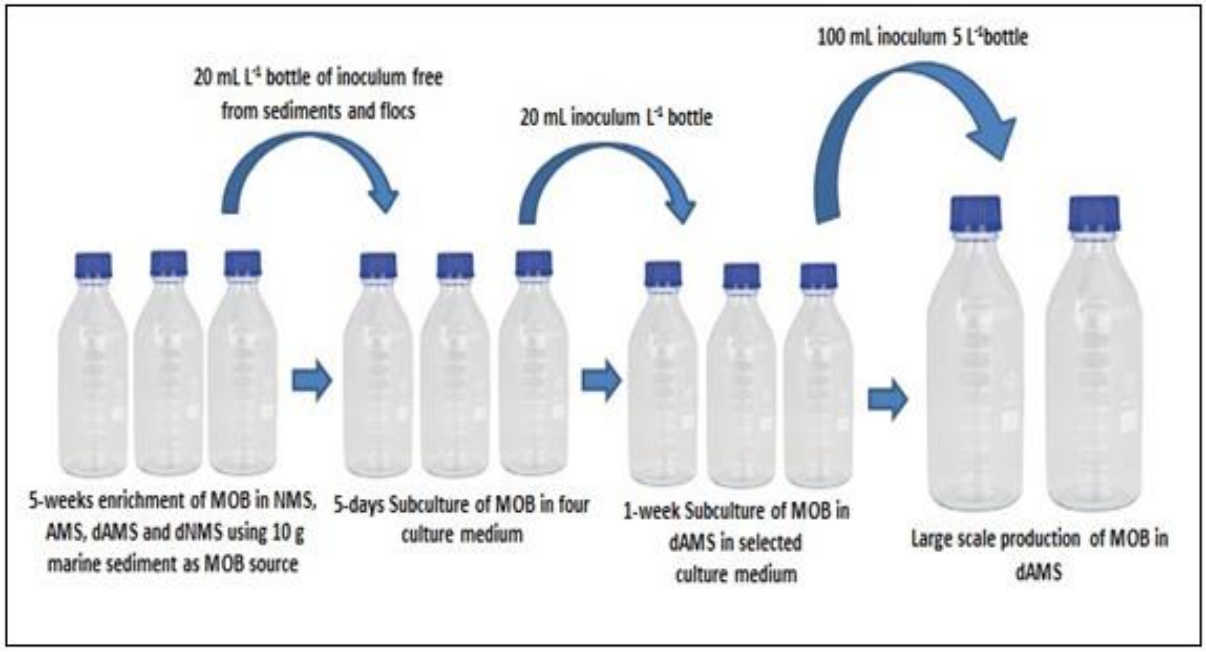


**Fig. 1.** General experimental flow of culture of MOB bacteria.

It is important to consider that our experiment only lasted for 6 days due to massive mortality. This experiment was repeated thrice in the hope to confirm the data and to grow the larvae till metamorphosis. However, the low quality of larvae (deformations) could be the reason; and based on the swab test and selective culture technique, *Vibrio*-attack interfered throughout the study. *Vibrio sp.* can hamper the larval and spat production (Dubert, *et al.*, 2017) and at 48 hours it lowers the survival of the mussel larvae where Irregular shaped and abnormal larvae are expected to occur (Casandra *et al.*, 2004). This latter finding agrees with the observed conditions of the larvae in this study. Moreover, stress and bad nutritional conditions of adult mussels can also cause a reduction of fertility with a decreasing number of gametes and modification in their biochemical composition which in turn affects the larval viability (Dame, 2012). Moreover, the fatty acid content of mussel egg is affected by the food quality of the adult mussel though the minimum level of PUFA is still passed on to the oocytes from the female mussel (Wacker, A., and von Elert, E. 2004).

**Fig. 2.** Experimental set-up for mussel larvae feeding test. Sixteen 10L Zugger bottles with 5L filtered seawater and up –flow aeration system.

This may explain the late development of trocophore to D-larvae and deformities of D-larvae that were used for the experiment. As observed, the deformed larvae had a convex hinge, protruding mantle, and incomplete shell and these abnormalities are due to poor quality and presence of contaminants (His, *et al.*, 1997). The produced D-larvae were either deformed or had a long transition from trocophore to D-larvae stage. There was also contamination of *Vibrio* sp. observed among those who were dealing with mussel larvae studies in the laboratory. *Vibrio* sp. may originate from the mussel brood-stock which serves as its reservoir and can cause 17% to 98% mussel larval death (Eggermont, *et al.*, 2014; Eggermont, *et al.*, 2017) or from the algae given (Simonsson, 2013).

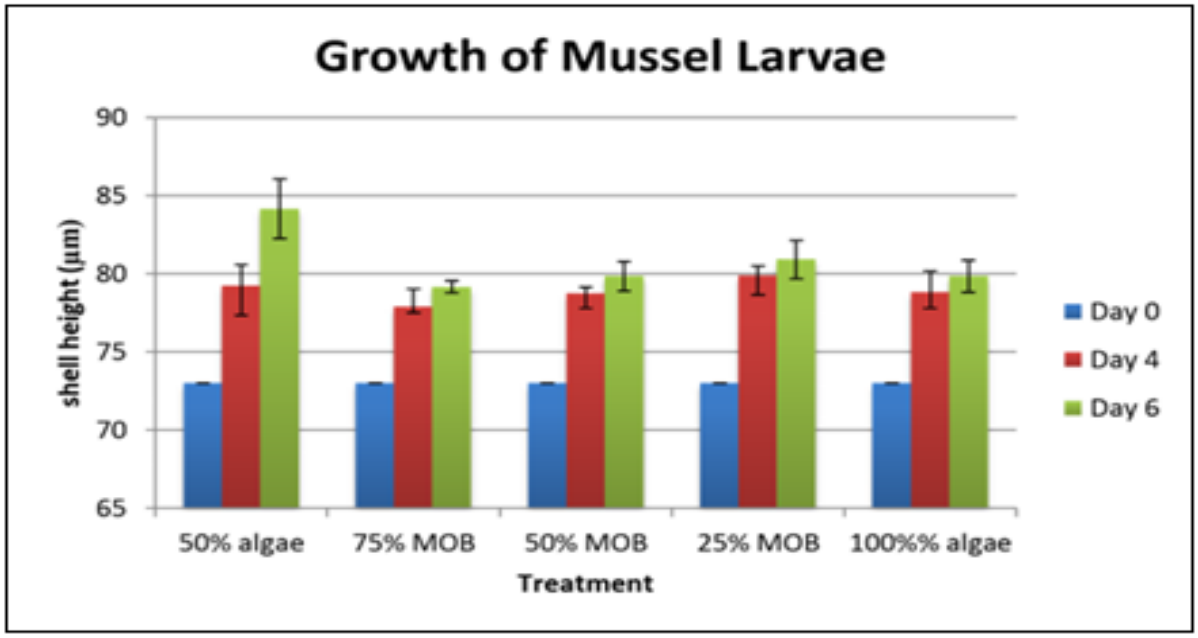


**Fig. 3.** Shell height measurement (80.7± 1.99 n=3; 50 larvae per replicate) of mussel larvae of different algae MOB concentration on dry weight basis.

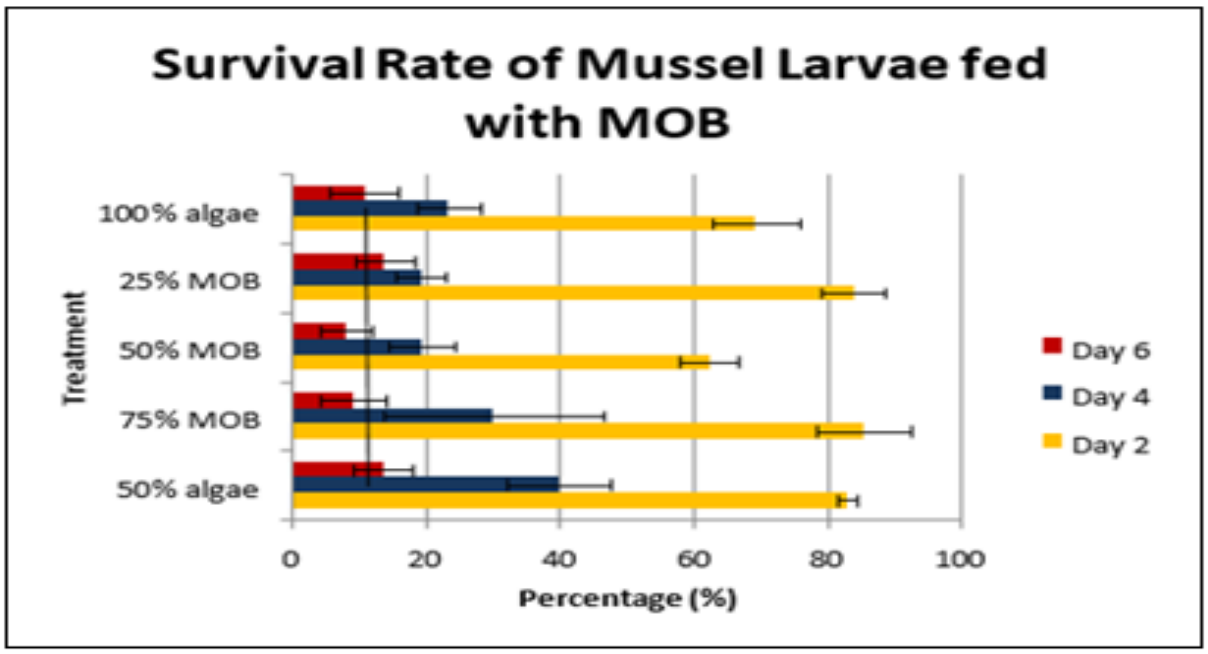


**Fig. 4.** Survival rate (mean 11.12±2.61%) of mussel larvae fed with different cell concentration of microalgae and MOB on dry weight basis.

## Conclusion

The effect of MOB on mussel larvae growth and survival is still considered as the same to the microalgae and with no negative effect. Considering MOB as feed replacement for mussel larvae diet cannot be discarded if not as the sole food for the mussel larvae. This can still help resolve the issue of microalgae higher production cost by diverting the demand to the MOB with a very low cost of production. A further full-term study is necessary to confirm the effect of MOB on the mussel larviculture. It is also recommended to conduct the research free from any viral or bacterial contamination of the area to prevent the massive death of larvae during the experiment.

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