The Open Access Israeli Journal of Aquaculture – Bamidgeh

As from **January 2010** The Israeli Journal of Aquaculture - Bamidgeh (IJA) will be published exclusively as **an on-line Open Access (OA)** quarterly accessible by all AquacultureHub (http://www.aquaculturehub.org) members and registered individuals and institutions. Please visit our website (http://siamb.org.il) for free registration form, further information and instructions.

This transformation from a subscription printed version to an on-line OA journal, aims at supporting the concept that scientific peer-reviewed publications should be made available to all, including those with limited resources. The OA IJA does not enforce author or subscription fees and will endeavor to obtain alternative sources of income to support this policy for as long as possible.

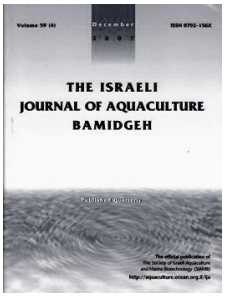
Published under auspices of
The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB),
University of Hawai'i at Mānoa Library
&
University of Hawai'i at Mānoa Aquaculture Program
in association with
AquacultureHub
http://www.aquaculturehub.org

ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

The Israeli Journal of Aquaculture - Bamidgeh, IJA_64.2012.732, 7 pages



Evaluation of Six Anesthetics for Use with the Mediterranean Mussel, *Mytilus galloprovincialis*

Ioannis N. Vatsos* and Panagiotis Angelidis

Ichthyology Laboratory, School of Veterinary Medicine, Aristotle University of Thessaloniki, TK 54124, P.O. Box 395, Greece

(Received 17.6.11, Accepted 21.8.11)

Key words: anesthesia, *Mytilus galloprovincialis*, 3-aminobenzoic acid

Abstract

An effective anesthetic scheme would significantly contribute to the investigation of disease in the Mediterranean mussel (*Mytilus galloprovincialis*), especially when biopsy is required. In this study, we evaluated the efficacy of six anesthetics: 2-phenoxyethanol (2 ml/l), MS222 (0.4 and 1 g/l), $MgCl_2$ (20 and 50 g/l), clove oil (1.5 ml/l), benzocaine (1.5 g/l), and 1-phenoxy-2-propanol (2.5 ml/l) when used to anesthetize Mediterranean mussel. For this purpose, 810 mussels were allocated to nine groups of 90 mussels each (three replicates of 30 individuals in each group). Each group was exposed to one of the above concentrations of anesthetics for 24 h, except for one group kept as the control. Mussels were considered anesthetized when they did not close their valves after tapping them with a pair of forceps. The most effective anesthesia was MS222 at the concentration of 1 g/l; it induced anesthesia in 58.8±1.92% of the exposed mussels with negligible mortality (5.7±5.8%) of the anesthetized mussels one week after anesthesia. Further, when using MS222 there was no correlation between the number of anesthetized mussels and temperature. However, the number significantly increased as the size of the mussels decreased. $MgCl_2$ at the concentration of 20 g/l resulted in notable retardation of valve movement.

* Corresponding author. Tel.: +30-2310-999880, fax: +30-2310-999871, e-mail: ivatsos@vet.auth.gr

Introduction

The Mediterranean mussel (*Mytilus galloprovincialis*) is one of the most commercially-important cultured shellfish in the Mediterranean area. Interest is increasing in the use of anesthetics to reduce stress in shellfish culture, for example, in oysters after the removal of pearls (Mamangkey et al., 2009). Collection of biological samples such as pieces of the mantle or hemolymph without killing a bivalve likewise requires effective anesthesia; anesthesia was used to remove mantle tissue from pearl oysters during selection of suitable donors for pearl spat production without inducing mortality (Acosta-Salmon et al., 2004). Effective anesthesia is also required in pathogenicity studies; magnesium chloride was used as an anesthetic to inject *Bonamia ostreae* into the heart cavity of European flat oysters (*Ostrea edulis*) for a cohabitation challenge (Lallias et al., 2008).

One of the main disease problems of the Mediterranean mussel *Mytilus galloprovincialis* is Marteiliosis, caused by the protozoon parasite *Marteilia refringens* (Virvilis et al., 2003; Karagiannis et al., 2006; Karagiannis and Angelidis, 2007; Anestis et al., 2010). Although this parasite does not induce mass mortality, it significantly affects the growth of affected mussels (Karagiannis et al., 2006; Anestis et al., 2010). Another disease of wild and cultured Mediterranean mussels, with yet unknown consequences, is hemic neoplasia (leukemia; Ciocan and Sunila, 2005), a condition probably related to environmental pollution. To investigate the effects of such diseases on mussels, initial screening of samples from live infected mussels is required. However, the collection of hemolymph or small tissue samples from live mussels is difficult as valves tend to firmly close when animals are stimulated while forcing them open can lead to fatal injury of the animal. Thus an effective anesthesia or deep relaxation is required.

In the present study, six anesthetic substances were evaluated for use with the Mediterranean mussel *Mytilus galloprovincialis*: 2-phenoxyethanol, MS222, magnesium chloride, clove oil, benzocaine, and 1-phenoxy-2-propanol. Many criteria can be used to assess the applicability of an anesthetic agent (Ross and Ross, 2008). Parameters taken into consideration in the present evaluation were (a) time required to induce anesthesia in a significant percentage of exposed mussels, (b) recovery period, and (c) induced mortality. After selecting the most effective substance, the effects of mussel size and water temperature on the effectiveness of the substance were investigated. In this paper, the terms 'anesthesia' and 'anesthetic' are used for all evaluated substances since it is difficult to distinguish between true 'anesthesia' and 'muscle relaxation' in bivalves (Norton et al., 1996).

Materials and Methods

Preparation of the anesthetic solutions. Six anesthetics were used in this study: 2-phenoxyethanol (Merck), MS222 (3-aminobenzoic acid, AppliChem), $MgCl_2$ (Mεrck), clove oil (Sigma), 1-phenoxy-2-propanol (Aldrich), and benzocaine (Fluka). Stock solutions of 2-phenoxyethanol, 1-phenoxy-2-propanol, and clove oil were prepared by mixing the substances with equal volumes of absolute ethanol, then appropriate volumes of these solutions were added to sea water and vigorously mixed to disperse the chemical into very small droplets. Stock solutions of MS222 and $MgCl_2$ were prepared by measuring appropriate quantities of the chemical and adding them directly to sea water. A stock solution of benzocaine was prepared by adding 100 g to one liter of absolute ethanol, adding an appropriate volume to sea water, and vigorously mixing the solution. The pH of the 0.4 g/l MS222 solution was 6 and that of the 1 g/l solution was 4. For all other solutions, the pH ranged 7.6-8.2.

Initial screening. To evaluate the anesthetic effect of the selected substances, 810 mussels (mean shell length 34±2.1 mm, mean total weight 3.4±0.28 g) were collected from a commercial farm in Thermaikos Gulf, Greece, and transferred in isothermal containers to the Ichthyology Laboratory at the Faculty of Veterinary Medicine. Upon arrival the mussels were scrubbed clean and randomly divided into nine groups, 90 mussels per group. The mussels of each group were placed into three 2-l plastic containers (3 replicates, 30 mussels per replicate) filled with sea water (salinity 35‰), that were constantly aerated to over 95% oxygen saturation, and maintained throughout

the experiment at 20±0.5°C. The mussels were left for two days to acclimatize and adhere to each other and/or the bottom of the containers via their byssi. After acclimation, the water of eight groups was replaced by sea water containing an anesthetic. In the three containers of the remaining group (control), the water was replaced by sea water containing no anesthetic. The number of anesthetized animals per container was recorded hourly for the first eight hours after addition of the anesthetic. A final measurement took place 24 h after addition of the anesthetic. Mussels whose valves remained open after gentle tapping three to ten times with a pair of forceps were considered anesthetized (Ross and Ross, 2008). The anesthetized mussels from each group were transferred to another container filled with sea water without anesthetic and left to recover (i.e., valves closed after tapping). The time at which the first and the last anesthetized mussel in each group recovered was recorded. Mussels that recovered were further monitored for one week and mortality was recorded (Table 1).

Table 1. Initial screening of six anesthetics for use with the Mediterranean mussel (*Mytilus galloprovincialis*).

Anesthetic and dose	*Anesthetized (%±SD)*		*Recovery time*		*Mortality after one week (%±SD)*
	at 8 h	*at 24 h*	*First to recover*	*Last to recover*	
Control	-	-	-	-	2.2±1.9^{a}
2-phenoxyethanol - 2 ml/l	10±3.3^{b}	42.2± 3.8^{b}	5-15 min	20-35 min	8.3±8.3^{a}
MS222 - 0.4 g/l	-	-	-	-	2.2±1.9^{a}
MS222 - 1 g/l	12.2± 1.9^{ab}	58.8±1.92^{c}	20-30 min	20-35 min	5.7±5.8^{a}
$MgCl_2$ - 20 g/l	-	-	-	-	2.2±1.9^{a}
$MgCl_2$ - 50 g/l	11.1±1.92^{b}	25.5±5.7^{a}	10-15 min	10-20 min	9.7±8.6^{a}
1-phenoxy-2-propanol - 2.5 ml/l	30±3.3^{c}	60±3.3^{c}	20-30 min	20-35 min	44.7±12.1^{b}
Clove oil - 1.5 ml/l	32.2±8.3^{c}	96.6± 3.3^{d}	-	-	100^{c}
Benzocaine - 1.5 g/l	22.2±1.92^{ac}	71.1±6.9^{c}	2-3 h	2.5-3.5 h	39.4±6.3^{b}

Different superscripts indicate significant differences between values in a column ($p \leq 0.05$).

Effect of mussel size. MS222 at 1 g/l induced the highest percentage of anesthetized mussels that recovered and had a mortality rate identical to that of the control. Thus, this treatment was further studied. Two size groups of 180 mussels, each, were formed: small (15±2 mm, 1.9±0.16 g) and large (48±2.2 mm, 4.8±0.3 g). The mussels in each group were randomly divided and placed into six 2-l containers (30 mussels per tank) filled with sea water and left to acclimatize as described above. After acclimation, MS222 at 1 g/l was added to three of the six containers of each size group, while no anesthetic was added to the remaining three containers (control). Anesthetized mussels, recovery, and mortality were recorded as above.

Effect of temperature. To test the effect of temperature on the efficacy of MS222-1 g/l, 360 mussels (48±2.2 mm, 4.8±0.3 g) were randomly divided into 12 plastic containers (30 mussels per container) filled with 2-l sea water. After acclimatization for two days, the mussels were divided into two groups containing 180 mussels, each, divided into six containers. The first group was kept at 18±0.5°C and the second group at 24±0.5°C. MS222-1 g/l was added to three containers of each group while the other three containers contained no anesthetic (control). Anesthetized mussels, recovery, and mortalities were recorded as above.

Statistical analysis. As evidenced by chi square test, results of the three replicates in each experimental group were very similar, thus, we pooled all replicates in each group to gain statistical power. Logistic regression analysis was used to model the effect of the substance on mussel anesthetization after 8 and 24 h of exposure (for each mussel, the outcome was 1 if it was anesthetized and 0 if not). The effectiveness of each substance was assessed by estimating Odds Ratios (OR) of anesthetization using two anesthetics at a time. Logistic regression analysis was also used to compare mortality one week after exposure (for each mussel, the outcome was 1 if it died and 0 if not). All statistical analyses were conducted using the statistical software program STATA (StataCorp. 2007. *Stata Statistical Software: Release 10*. StataCorp LP, College Station, TX).

Results

Initial screening. With the exception of mussels exposed to $MgCl_2$-20 g/l and the control group, all mussels that were not anesthetized remained closed throughout the exposure period. Mussels in the control group exhibited active valve movements (they opened and closed) throughout the 24 h experimental period and responded immediately after tapping two or three times with a pair of forceps by closing their valves. Though it seems that benzocaine, 1-phenoxy-2-propanol, and clove oil were most effective in anesthetizing mussels, they also statistically increased mortality. In contrast, there was no significant difference in mortality between the MS222-1 g/l and control groups (p = 0.29). Further, the recovery period for benzocaine was longer than for all other anesthetics. $MgCl_2$ at 50 g/l had a low anesthetic effect and at 20 g/l had no anesthetic effect; almost all mussels exposed to $MgCl_2$-20 g/l had open valves and the feet extended well outside the valves after 8 h exposure although, when stimulated by a pair of forceps, they exhibited notable slowness in response and valves closed only after at least ten taps. All mussels that appeared anesthetized during exposure to clove oil never recovered and died within 24 h. Thus, the results of this group were not included in the assessment of induced anesthetic effects.

Based on the percentage of anesthetized mussels, the recovery period, and mortality, mussels exposed to MS222-1 g/l were further maintained in this solution until almost all were anesthetized. The percentages of mussels anesthetized were 82.3±1.9% after 48 h, 83.3±3.3% after 72 h, 96.6±3.3% after 96 h, and 98.8±1.9% after 120 h. No significant mortality was observed in mussels that recovered one week after this prolonged exposure period; cumulative mortality after 120 h of exposure was 9±5.2%.

Effect of mussel size and temperature. After 24 h of exposure, the percentage of anesthetized mussels was significantly higher in the smaller size group than in the larger (Table 2). Mussel size was a significant predictor of anesthetization in the logistic regression model (p<0.001) and the respective OR estimate of anesthetization in large compared to small mussels was 0.33. The respective OR at 8 h was 0.578, not statistically different from 1 (p = 0.25). There was no significant effect of temperature on the number of mussels anesthetized by MS222-1 g/l.

Table 2. Effect of mussel size and temperature on the anesthetic effect of MS222-1 g/l on Mediterranean mussels.

	Anesthetized (%±SD)		*Recovery time*		*Mortality after one week (%±SD)*
	at 8 h	*at 24 h*	*First to recover*	*Last to recover*	
Mussel size					
15±2 mm SD					
MS222	14.4±5	65.5±8.3[a]	15 -25 min	20 -30 min	-
Control	-	-	-	-	-
48±2.2 mm SD					
MS222	8.8±1.9	38.8±5[b]	20 -25 min	20 -30 min	-
Control	-	-	-	-	-
Temperature					
24±0.5°C					
MS222	8.8±1.9	38.8±5	15 -25 min	25 -35 min	2.2±1.9
Control	-	-	-	-	5.8±5.2
18±0.5°C					
MS222	10±3.3	33.3±6.6	25 -35 min	25 -30 min	2.2±1.9
Control	-	-	-	-	-

Different superscripts indicate significant differences between values in a column (p≤0.05).

Discussion

The study of anesthesia in shellfish is limited because anesthetics are not routinely used in their production cycles. On the other hand, effective anesthesia is often required in fish production, for example, prior to grading or during vaccination and sampling. Recent increased interest in the pathology of major diseases in Mediterranean mussels has created the need for effective anesthesia to collect tissue samples (mantle, gills, hemolymph, gonads) while maintaining animals alive for further investigation. The present study investigated six substances known to have anesthetic effects on other shellfish species (Norton et al., 1996; Mamangkey et al., 2009; Suquet et al., 2009; Vatsos and Angelidis, 2010).

When bivalves such as the Mediterranean mussel encounter adverse and harmful environmental conditions such as handling or toxicants, they usually respond with prolonged closure of the valve and a lower filtration rate (El-Shenawy et al., 2001). Therefore, factors that can inflict stress to bivalves, such as handling or even the anesthetic itself, may affect the effectiveness of an anesthetic, especially the exposure period required to obtain the desired anesthesia (Mills et al., 1997). Authors address this issue either by forcing the bivalve to remain open during exposure by inserting a small piece of wood inside the valve (Norton et al., 1996) or by stimulating the filtration function in an attempt to increase the uptake of the anesthetic (Suquet et al., 2009). In the present study, no such approach was attempted, as our aim was to compare the selected substances in simulated natural conditions and assess the behavior of the organisms during exposure.

MS222 at the concentration of 1 g/l was the most effective substance. In fish, MS222 and benzocaine, substances with similar structures, are absorbed by the gills and produce general anesthesia by suppressing neural signal transmission from the periphery to the central nervous system (Zahl et al., 2009). A similar mode of action was reported for 1-phenoxy-2-propanol in gastropods, where the substance also reduced the force of muscle contraction (Wyeth et al., 2009). 2-phenoxyethanol induces anesthesia possibly by inhibiting the activity of excitatory N-methyl-D-aspartate receptors (Mußhoff et al., 1999). Clove oil and magnesium chloride are not true anesthetics in bivalves (Ross and Ross, 2008); the first acts as a muscle relaxant, while the second interferes with muscle contractibility (Suquet et al., 2009).

In our study, the period of exposure required to induce a notable number of anesthetized mussels was particularly long. A preliminary study of *M. galloprovincialis* under similar conditions indicated that 24 h exposure to 2 ml/l 2-phenoxyethanol (the concentration of 2-phenoxyethanol that resulted in the highest percentage of anesthetized mussels) induced anesthesia in approximately 67% of the exposed mussels (Vatsos and Angelidis, 2010). In the present study, even after 24 h of exposure, the percentages of anesthetized mussels were well below those reported for Pacific oyster, where almost 100% were anesthetized after exposure to similar concentrations of magnesium chloride (50-72 g/l) for 16 h (Suquet et al., 2009). Similarly, approximately 96% of silver-lip pearl oyster (*Pinctada maxima*) were anesthetized after only 14 min of exposure to 3 ml/l 2-phenoxyethanol (Mamangkey et al., 2009) and a high percentage of pearl oysters (*Pinctada albina*) were anesthetized after 1 h exposure to 2.5 ml/l 1-phenoxy-2-propanol (Norton et al., 1996) but, in these two studies, valves were forced to remain open during exposure by insertion of wedges. In the present study, 96.6±3.3% of the population were anesthetized only after 96 h of exposure to MS222-1 g/l, possibly because of stress induced prior to or during anesthesia that caused the mussels to keep their valves closed for a particularly long period. Since the control mussels exhibited normal behavior during the exposure period, factors related to the anesthetic were probably responsible for this phenomenon. Even this prolonged exposure was not accompanied by significant mortalities.

The use of MS222-1 g/l in our study did not cause increased mortality as when the same concentration of MS222 (pH 4), was used with pearl oysters (Norton et al., 1996). In that study, the mortality was associated with the low pH of the anesthetic solution. The different tolerance of Mediterranean mussel to short-term exposure to low pH could explain the low mortality observed in our study, however, this needs further confirmation. Clove oil resulted in almost 100% mortality during the first 24 h. Clove oil was also associated with increased mortality in silver-lip pearl oysters (Mamangkey et al., 2009). However, clove oil at the examined concentration was proposed for other mollusks such as abalone (*Haliotis lavigata, H. rubra*; Ross and Ross, 2008).

In the present study, the percentage of anesthetized animals was greater in smaller mussels but was not affected by temperature. There are contradicting reports on the effects of these two factors on anesthesia in fish. For most fish species, induction time is lower and effectiveness is greater in small fish and in those maintained at higher temperatures, possibly because basal metabolism is higher in small fish and in high

temperatures, although other factors may be involved (Zahl et al., 2009). There are also contradicting reports on the effects of temperature and body size on anesthesia in bivalves. As in the present study, the number of anesthetized Pacific oysters in smaller animals was greater than in large (Suquet et al., 2009) but, contrary to our study, there was no effect of size on the effectiveness of any of the anesthetics examined in pearl oysters (Norton et al., 1996). Also in contrast to our results, there was a positive correlation between the number of anesthetized oysters and temperature (Suquet et al., 2009). In bivalves, the metabolic and filtration rates increase as the temperature increases (Yukihira et al., 2000). Smaller bivalves exhibit increased metabolism (Yukihira et al., 1998) and filtration rates when expressed in relation to their dry meat weight (Walne, 1972). However, the effects of temperature and body size on the effectiveness of anesthetic substances may depend on species-related factors and, probably, properties of the anesthetic.

The present study indicates that MS222 at the concentration of 1 g/l can be used as a relatively safe anesthetic for the Mediterranean mussel, but only after prolonged exposure. This substance can be readily dissolved in sea water and does not require vigorous mixing prior to use, as does 2-phenoxyethanol and 1-phenoxy-2-propanol. The use of magnesium chloride at the concentration of 20 g/l resulted in significant retardation of the response of mussels to external stimulation (i.e., valves closed only after repeated tapping). Gaping of valves after use of $MgCl_2$ at 30-50 g/l has been observed in many bivalve species (Heasman et al., 1995). This is a desired effect when deep relaxation of the bivalves is required to reduce the adverse effects of stressful conditions, for example, during observation and selection of individuals for breeding (Heasman, et al., 1995).

References

Acosta-Salmon H., Martinez-Fernandez E. and P.C. Southgate, 2004. A new approach to pearl oyster broodstock selection: can saibo donors be used as future broodstock? *Aquaculture*, 231:205-214.

Anestis A., Pörtner H.O., Karagiannis D., Angelidis P., Staikou A., and B. Michaelidis, 2010. Response of *Mytilus galloprovincialis* (L.) to increasing seawater temperature and to marteliosis: metabolic and physiological parameters. *Comp. Biochem. Physiol. Part A,* 156:57-66.

Ciocan C. and I. Sunila, 2005. Disseminated neoplasia in blue mussels, *Mytilus galloprovincialis*, from the Black Sea, Romania. *Mar. Pollut. Bull.,* 50(11):1335-1339.

El-Shenawy N.S., Greenwood R., Nabil Z.I., Abdel-Nabi I.M. and R.A. Hanna, 2001. Valve movement behaviour and byssal formation of the mussel, *Mytilus edulis*, in relation to environmental toxins. *Egypt. J. Biol.,* 3:63-71.

Heasman M.P., O'Connor W.A. and A.W.J. Frazer, 1995. Induction of anaesthesia in the commercial scallop, *Pecten fumatus* Reeve. *Aquaculture* 131:231-238.

Karagiannis D. and P. Angelidis, 2007. Infection of cultured mussels *Mytilus galloprovincialis* by the protozoan *Marteilia* sp. in the Thermaikos Gulf (N. Greece). *Bull. Eur. Assoc. Fish Pathol.,* 27(4):131-141.

Karagiannis D., Angelidis P., Staikou A. and B. Michaelidis, 2006. The effect of *Marteilia* sp. infestation on the scope for growth of mussels *Mytilus galloprovinciallis* L. p. 541. In: *Conf. Proc. EAS Conference*, Florence, Italy.

Lallias D., Arzul I., Heurtebise S., Ferrand Chollet B., Robert M., Beaumont A.R., Boudry P., Morga I. and S. Lapegue, 2008. *Bonamia ostreae*-induced mortalities in one-year old European flat oysters *Ostrea edulis*: experimental infection by cohabitation challenge. *Aquat. Living Resour.,* 21:423-439.

Mamangkey N.G.F., Acosta-Salmon H. and P.C. Southgate, 2009. Use of anaesthetics with the silver-lip pearl oyster, *Pinctada maxima* (Jameson). *Aquaculture,* 288:280-284.

Mills D., Tlili A. and J. Norton, 1997. Large-scale anesthesia of the silver-lip pearl oyster, *Pinctada maxima* Jameson. *J. Shellfish Res.,* 16:573-574.

Mußhoff U., Madeja M., Binding N., Witting U. and E.J. Speckmann, 1999. Effects of 2-phenoxyethanol on N-methyl-D-aspartate (NMDA) receptor-mediated ion currents. *Arch. Toxicol.*, 73:55-59.

Norton J.H., Dashorst M., Lansky T.M. and R.J. Mayer, 1996. An evaluation of some relaxants for use with pearl oysters. *Aquaculture,* 144:39-52.

Ross L.G. and B. Ross, 2008. *Anaesthetics and Sedative Techniques for Aquatic Animals*. Blackwell Publ. 240 pp.

Suquet M., De Kermoysan G., Gonzalez Araya R., Queau I., Lebrun L., Le Souchu P. and C. Mingant, 2009. Anaesthesia in Pacific oyster, *Crassostrea gigas*. *Aquat. Living Resour.*, 22:29-34.

Vatsos I.N. and P. Angelidis, 2010. Use of 2-phenoxyethanol, magnesium chloride and MS222 as anaesthetics in mussels *Mytilus galloprovincialis*. pp. 181-184. In: *Conf. Proc. 14th Pan-Hellenic Conference of Ichthyologists*, 6-9 May 2010. Pereus, Greece.

Virvilis C., Angelidis P. and G. Photis, 2003. Presence of the parasite *Marteilia* sp. in the shellfish of the Thermaikos Gulf in northern Greece. *Bull. Eur. Assoc. Fish Pathol.,* 23(4):157-162.

Walne P.R., 1972. The influence of current speed, body size and water temperature on the filtration rate of five species of bivalves. *J. Mar. Biol. Assoc. UK,* 52:345-374.

Wyeth R.C., Croll R.P., Dennis Willows A.O. and A.N. Spencer, 2009. 1-Phenoxy-2-propanol is a useful anaesthetic for gastropods used in neurophysiology. *J. Neurosci. Meth.,* 176:121-128.

Yukihira H., Klumpp D.W. and J.S. Lucas, 1998. Effects of body size on suspension feeding and energy budgets of the pearl oysters *Pinctada margaritifera* and *P. maxima. Mar. Ecol. Prog. Ser*., 170:119-130.

Yukihira H., Lucas J.S. and D.W. Klumpp, 2000. Comparative effects of temperature on suspension feeding and energy budgets of the pearl oysters *Pinctada margaritifera* and *P. maxima*. *Mar. Ecol. Prog. Ser.,* 95:179-188.

Zahl I.H., Kiessling A., Samuelsen O.B. and M.K. Hansen, 2009. Anaesthesia of Atlantic cod (*Gadus morhua*) — Effect of pre-anaesthetic sedation, and importance of body weight, temperature and stress. *Aquaculture,* 295:52-59.