Thesis for the Degree of Master of Fisheries Science

# The histological effects of a bioenergy water on degenerated adductor muscles in cultured juvenile abalone, *Haliotis discus hannai*

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# Tables of Contents

Tables of contents ····· i

List of Figures ····· iv

List of Tables ····· vii

Abstract ····· viii

I. Introduction ····· 1

II. Materials and Methods ····· 7

1. Study site ····· 7

2. Experiment design ····· 7

3. Sample collection ····· 8

4. Histological methods ····· 8

4.1 Dissection ····· 9

4.2 Fixation and Re-fixation ····· 9

4.3 Tissue processing ····· 9

4.4 Wax embedding ····· 9

4.5 Sectioning ····· 10

4.6 Staining ····· 10

4.7 Mounting ····· 12

5. Photography ····· 12

6. Anatomical examination ····· 12
7. Histological evaluation ····· 12
III. Results ····· 15
1. First sample ····· 15
1.1 Anatomical measurement ····· 15
2. Second Sample ····· 20
2.1 Anatomical measurement ····· 20
2.2 Juveniles' adductor muscle histology evaluation ····· 22
2.2.1 Abalones in general water group ····· 22
2.2.2 Abalone in bio-energy water group ····· 22
3. Third Sampling ····· 25
3.1 Anatomical measurement ····· 25
3.2 Juveniles' adductor muscle histology evaluation ····· 27
3.2.1 Abalone in general water group ····· 27
3.2.2 Abalone in bio-energy water group ····· 27
4 Fourth sampling ····· 30
4.1 Anatomical measurement ····· 30
4.2 Juveniles' adductor muscle histology evaluation ····· 32
4.2.1 Abalone in general water group ····· 32
4.2.2 Abalone in bio-energy group ····· 32
5 Fifth sampling ····· 35

5.1 Anatomical measurement ····· 35
5.2 Juveniles' adductor muscle histology evaluation ····· 37
5.2.1 Abalone in general water group ····· 37
5.2.2 Abalone in bio-energy group ····· 37
IV. Discussion ····· 41
V. Acknowledgement ····· 47
VI. References ····· 49

# List of Figures

Fig. 1. Changes in morphological features of muscle degeneration of adductor muscles. (A: Magnified X400 and B: Magnified X1000.H&E __50 µm).Cellular swelling in adductor muscle fibrils, muscle vacuolization of the adductor muscle fibres (arrows). Cellular atrophy, degenerative muscle fibrils, myonecrosis (elliptical circles) in muscle fibres and loss of muscle fibres in adductor muscle are shown in both figures (1-A, 1-B. H&E __50 µm)…….. 14

Fig. 2. Photos of the adductor muscle of abalone before applying bio-energy water (1-8: 400 times). Vacuolization, lesion, atrophy and muscle fiber loss (arrows) are clearly visible in images 1-8. In image (9: X1000 magnified) lesion in muscle histology (elliptical circle) is clearly shown (H&E_50µm)……19

Fig. 3. Photos of the adductor muscle of abalone in general water after one week (1-8: 400 times). Vacuolization, vitrification, swelling, thin diameter, lesion, atrophy and muscle fiber loss (arrows) are clearly visible in images 1-8. In image (9: X1000 magnified) lesion in muscle histology (elliptical circle) is clearly shown (H&E_50 µm)……23

Fig. 4. Photos of the adductor muscle of abalone in bio-energy water after one week (1-8: 400 times). The muscle fiber grew thicker, vacuolization and swelling gradually decreased (arrows) in images (1-8) as compared to the first sample. In image (9: X1000

magnified) improvement in adductor muscle histology (elliptical circle) is shown (H&E __50 μm)……24

Fig. 5. Photos of the adductor muscle of abalone in general water after two weeks (1-8: 400 times).Muscle fiber lesion of hypertrophy, Vacuolization, swelling and necrosis appear. Vacuolization, lesion, atrophy and muscle fiber loss (arrows) are clearly visible in images 1-8. In image (9: X1000 magnified) lesion in muscle histology (elliptical circle) is clearly shown (H&E_50 μm)……28

Fig. 6. Photos of adductor muscle of abalone in bioenergy water after two weeks (1-8: 400 times). Lesions, swelling of the adductor muscle, vitrification (hyalinization) and vacuolization of the muscles disappeared (arrows). Muscle fiber thickness and diameter improved. In image (9: 1000 times enlarged) adductor muscle recovery (elliptical circles) can clearly be observed (H&E__50μm)……29

Fig. 7. Photos of the adductor muscle of abalone in general water after three weeks (1-8: 400 times). Vacuolization, lesion, hypertrophy, atrophy and muscle fiber loss (arrows) are clearly visible in images 1-8. In image (9: X1000 magnified) lesion in muscle histology (elliptical circle) is clearly shown (H&E_50μm)…….33

Fig. 8. Photos of adductor muscle of abalone in bio-energy water after three weeks (1-8: 400 times).Shell mounting portion of the adductor muscle, vitrification (hyalinization), vacuoles in cytoplasm , atrophy and muscle fiber loss improved significantly (arrows). Swelling and vacuolization in the muscle fiber almost disappeared. In image (9: 1000 times enlarged) muscle lesion (elliptical circle) also disappeared (H&E __50 μm)……34

Fig. 9. Photos of the adductor muscle of abalone in general water after four weeks (1-8: 400 times). Vacuolization, lesion, hypertrophy, atrophy and muscle fiber loss (arrows) are clearly visible in images 1-8. In image (9: X1000 magnified) lesion in muscle histology (elliptical circle) is clearly shown (H&E_50μm)……38

Fig. 10. Photos of adductor muscle of abalone in bio-energy water after four weeks (1-8: 400 times).The muscle fiber and muscular bundle recovered in all individuals of this sample (n=8). Increase in muscle fiber diameter, no vacuolization or swelling of the muscle fiber (arrows) is clearly shown. In image (9:1000 times enlarged) the complete adductor muscle fiber recovery (elliptical circle) is visible (H&E __50 μm)……...39

Fig. 11. Adductor muscle recovery in juvenile abalone in control group (general water, 0 weeks) compared with that of treatment groups (bio-energy water, 1-4 weeks).The area of muscle in adductor muscle was calculated with 400 magnification photograph.....…...40

# List of Tables

Table 1. Body weight and shell measurement of abalones in first sample at 0 weeks.....16

Table 2. Body weight and shell measurement of abalones in second sample at 1 weeks..21

Table 3. Body weight and shell measurement of abalones in third sample at 2 weeks….26

Table 4. Body weight and shell measurement of abalones in fourth sample at 3 weeks...31

Table 5. Body weight and shell measurement of abalones in fifth sample at 4 weeks….36

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## Abstract

This study was conducted to examine the recovery of degenerated adductor muscles in juvenile abalone (*Haliotis discus hannai*) which were cultured in bioenergy water for four weeks. Juvenile abalones having average total weight 40.76±24.23 g, shell weight 10.99±7.14 g, shell length 4.87±0.64 cm, shell width 0.96±0.20 cm, and height 3.37±0.48 cm, were fetched from an abalone farm, south Jeola Province Wando County, Republic of Korea. Juvenile abalones were divided into two groups, i.e., treatment and control group. General sea water was used in control whereas bioenergy water was used in treatment groups. Five samples, in all, were collected randomly with (n=8) individuals in each

sampling. Juvenile abalones were dissected, fixed and refixed (in 10%formalin). Adductor muscles were chopped, then dehydrated for tissue processing (in 70-100% ethyl alcohol) and embedded with paraffin wax (58-62ºC). Thin (4μm) ribbons of embedded tissues were obtained using rotatory microtome which were then stained with Haematoxylin and Eosin (H&E method) and preceded for light microscopy finally. In the first sample (0 weeks), adductor muscles showed atrophy (necrosis).In the second sample (1 week after applying bioenergy water), there was a great improvement in muscle histology. Muscle fiber histology recovered completely in the third sample (2 weeks after applying bioenergy water). Lesions (swelling) disappeared in the fourth and the fifth samples too (3 and 4 weeks after applying bioenergy water).The adductor muscles recovered because of immunopotentiating characteristics of bioenergy water that sustained its normal structure. Results of this study suggest that bioenergy water can cure the degenerated adductor muscles of juvenile abalone**.**

Keywords: Abalone, *Haliotis discus hannai***,** Bioenergy water, Adductor muscles, Immunity.

# I. Introduction

Abalones (marine snails or gastropods) belong to phylum Mollusca, class Gastropoda, sub-class Orthogastropoda, order Vetigastropoda, super family Pleurotomarioidea, family Haliotidae and genus Haliotis (L.R Cox 1960, Ponder 2000). Haliotis generally means "sea ear" and refers to the ear-like shape of the abalone shell (Harry Keith Gorfine, April 2002). Abalones exist worldwide and are found in tropical, sub-tropical, warm temperate regions in both northern and southern hemispheres. The temperate zone contains large size abalones, whereas the small size abalones inhabit cold and tropical zones (Geiger 1999, Lindberg, 1992).

Abalones have a smooth flattened spiral shaped shell that protects it from strong waves of the sea (Voltzow 1990). Respiratory pores are found on the shell (outer left margin) that carries out oxygenation in gills, excretion of renal waste and discharge of gametes (Bames 1986). At present, over 100 species of abalones have been reported to exist in the world oceans (Hahn 1989c, FAO 1990). Twenty-six(26) Haliotis species are commercially important and 16 species are being cultured in the world(Paul Leighton,2008).The optimum conditions for abalone, *Haliotis discus hannai*, culture are: water temperature 20 °C, salinity around 30 ppt, DO more than 4ml/L, pH around 8.0 –

8.3 (thearith, 2016). They are said to be sexually mature when, both, male and female abalone have a shell length of approximately 60 mm (Nie, 1992).

Abalone *(Haliotis discus hannai)* is successfully being cultured in Korea, China and Japan (FAO 1990). Farmed abalone production has increased approximately up to 750% in less than a decade because of depletion in abalone capture since 1970s (Peter A. Cook, 2016). China, producing 87% of total global production of abalone, has the largest market for dried abalone meat and shell products (Peter A. Cook, 2016). Korea,SouthAfrica,Chile,Australia,USA,Japan,Taiwan,Newzealand,Thialand,Philippines e etcetera are among major contributors of abalone production in international market respectively (FAO2016, Peter A. Cook, 2016).

In culture system, water quality, temperature, dissolved oxygen, pH, salinity, nitrogenous wastes, environmental pollutants, food availability, stress and stocking density are factors that inhibit growth rate and also affect immune system of abalone (Fisher 1986). Temperature fluctuation changes the physiology and immune functions of ectothermic organisms, eventually causing mass mortalities (Dang etal., 2012; Hooper et al; 2014). High mortality of fish and shellfish was reported in July, 2012 in Korea because of rise in sea temperature (Lee et al., 2013). Any fluctuations in dissolved oxygen (DO) reduces the food intake that results in poor growth (Harris et al., 1999a).Salinity between 25-45 ppt is bearable, but salinity lower than this causes death (Shumway 1977). High ammonia concentration causes stress, perturbs cellular biochemistry and physiology in marine

organisms (Huchette et al., 2003b). High stocking density decreases growth because of competition for shelter and food (Mgaya & Mercer 1995).

Adductor muscle in abalone carries out body movements and helps the body attach on to a substratum. It also balances the body, helps in locomotion and food manipulation (Trueman & Brown 1985, Frescura 1990). Increased stress and stressors cause decreased immune functional capacities that retard growth, leading to disease outbreaks and bacterial infection in the farmed abalone. (Cheng et al., 2004).Oxidative stress, reactive oxygen agents and elevated temperature disrupt adductor muscle tissues causing myofibrillar necrosis or premature death of abalone (Kim et al., 2014).

The role of water in cellular structure and function is indispensible. Until now there is no report available about the role of water in relation with immunity. This study strongly focused on the critical role of water in immunity which is a new innovative concept. Water, with a molecular formula $H_2O$, has peculiar properties because of hydrogen bonding present in it. The natural chemical processes of protonation/de protonation of hydrogen atoms within water molecule controls cellular biochemistry (Martin Chaplin, 2001).Water plays a pivotal role in structure and function of biomolecules (Smolin et al., 2005). Seventy percent (70%) of human body comprises water. Breathing, metabolism, digestion and detoxification (i.e. immunity) in the living body are important physiological functions performed by water. Water regulates body temperature and maintains osmotic balance within the cell and its external environment. Cellular structure, its cohesion and shape is maintained by water (Roganovic, 2011).

Unfortunately, water and its role in the cellular architecture and maintenance of immunity has superficially been studied. Water, to date, has been considered a simple medium and merely a solvent present in the cell. In fact, the life and death of a cell and all organisms (plants and animals) depends upon water completely. Water is the reality of immunity since it is only water in the cell or in the entire organisms that restores immune functions. An obsolete biased approach towards water and its role in reinstating immunity has been applied, either because of lack of knowledge or prejudice in the realm of science that is not logically based (Chankakada Mak 2016). Today pathogens are said to be the sole cause of disease outbreak, but water, the most abundant molecule in the body and the most essential for all forms of life, is not considered important at all. Any kind of water disruption within or outside the cell changes the structure of biomolecules which, then, leads to pathological diseases (Robert M. Davidson Ann Lauritzen and Stephanie Seneff, 2013).

Bio-energy water, introduced by DM Bio Company Limited Korea, is an innovatively upgraded drinking water which has health beneficial values, particularly those of immune recovering and immune activating in plants, human beings and animals equally. This water does not contain any additives and is obtained by natural energy (light/solar energy having a wavelength of 4- 14μm).This light energy (4- 14μm) absorbs in the water that strengthens the natural hydrogen bonding in the water molecule, imparts long lasting biochemical and physiological effects in the cell by sustaining the original structure of biomolecules and their relative positioning in the tissues. This water is obtained by

passing water through a patented purifying instrument created and developed by DM Bio Company Limited Korea.

In this study it was hypothesized that bio-energy water can heal degenerated adductor muscles of abalone. Therefore, the histopathological features of adductor muscles of abalone were investigated after treating it with bio-energy water so as to analyse the recovering effects of bioenergy water on the degenerated adductor muscles in abalone, *Haliotis discus hannai*.

# Objective

The main objective of this study is to investigate the healing ability of bio-energy water on the histology of adductor muscles in cultured juvenile abalone *(Haliotis discus hannai)*.

The specific objectives include:

Examine the recovering effects of bioenergy water in adductor muscle histology.

Identify histological and structural alteration in semi quantitative method.

Compare the recovery of affected adductor muscle after using, both, general and bio-energy water.

# II. Materials and Methods

## 1. Study site

### 1.1 South Jeollanam-do Wando County

Both male and female abalones *(Haliotis discus hannai)* were fetched from different clinically healthy cement tank culture farms in South Jeollanam-do Wando County. This study was done at Laboratory of Fish and Shellfish Pathology (LFSP), Department of Aquatic Life Medicine, Pukyong National University, Republic of Korea. Juvenile abalones having average total weight 40.76±24.23 g, shell weight 10.99±7.14 g, shell length 4.87±0.64 cm, shell width 0.96±0.20 cm, and height 3.37±0.48 cm were selected for this study. This study focused on investigating histological alteration in adductor muscles of juvenile abalone*, Haliotis discus hannai,* by using bio-energy water.

## 2. Experiment design

Abalones were divided into two groups. The same conditions for aeration, temperature, salinity, pH and DO were maintained during the experiment.

First group was cultured in general water and the second group was cultured in bio-energy water. Both groups were kept under fasting condition during this experiment.

Abalones were cultured in circular plastic water tank for four weeks from December 1st, 2016 to January 2nd, 2016. Abalones were reared in two circular plastic water tanks, by using the flow through system where same source of water from the sea was used in South Jeollanam-do Wando County. The environmental conditions in both the culture tanks were maintained at same conditions for aeration, water temperature, salinity, pH, DO, and ammonia.

## 3. Sample collection

Five samples, in all, were collected from total amount of abalones. Eight (n=8) abalones were collected for histological studies before starting experiment. Then, during the sample collection, 8 abalones from general water and 8 abalones from that of bio-energy water were collected per sampling by random collection.

## 4. Histological methods

### 4.1 Dissection

The body weight, length, width, and height were measured by electronic scale and plastic ruler, and then dissected in the laboratory. Dissection was done with help of forceps and scissors. The abalone shell was removed out, so that the abalone adductor muscle could appear clearly. The pictures were taken by using digital camera (Olympus E-P2-Japan).

### 4.2 Fixation and Re-fixation

The adductor muscle was fixed in Bouin's solution. After 24 hours, those samples were cut in small piece by sharp blade and put in labelled cassettes. All the labelled cassettes samples were re-fixed in 10% buffered formalin solution.

### 4.3 Tissue processing

All the collected tissue samples were passed through a series of solvents for paraffin wax embedding (paraffin wax at 58 – 62 $^{0}$C). Tissues were washed and dehydrated through ethyl alcoholic grades (70%, 80%, 90%, 95%, 100%, 100%, and 100%). All samples were cleaned for 21 hours in xylene and 30 minutes before paraffin wax embedding.

### 4.4 Wax embedding

The samples of adductor muscle were embedded with paraffin wax at 58-62 $^{0}$C.

### 4.5 Sectioning

A part of embedded blocks of each tissue sample was sliced into 4 µm sectioned ribbons using a rotary type microtome (Reichert – Jung 820, Leica, Germany). The sectioned ribbons were floated on the alcohol bath (10%) and then in warm water bath at 50 $^{0}$C to flatten out the sectioned ribbons. The sectioned ribbons were carefully collected on to a glass slide and were allowed to dry fully and flatten at 40 $^{0}$C for 12 hours before proceeding to Hematoxylin and Eosin (H & E) staining.

### 4.6 Staining

Hematoxylin and Eosin (H&E) staining method was used to stain the tissue section. The following consecutive steps were followed to perform H&E staining:

Xylene (1) - 3 minutes

Xylene (2) - 3 minutes

Xylene (3) - 3 minutes

Ethyl alcohol 100% - 1 minute

Ethyl alcohol 95% - 1 minute

Ethyl alcohol 90% - 1 minute

Ethyl alcohol 80% - 1 minute

Ethyl alcohol 70% - 1 minute

Washing with flowing tap water- 10 minutes

Hematoxylin - 3 minutes

Washing with tap water - 1 minute

HCL (Acid alcohol) - 2 times dipping

Washing with tap water - 1 minute

Ammonia water - 4 times dipping

Washing with flowing tap water - 15 minutes

Eosin - 2 minutes

Ethyl alcohol 70 - 80% - 4 times dipping

Ethyl alcohol 90% - 1 minute

Ethyl alcohol 95% - 1minute

Ethyl alcohol 100% - 1 minute

Ethyl alcohol 100% - 1 minute

Xylene + Alcohol - 3 minutes

Xylene (1) - 3 minutes

Xylene (2) - 3 minutes

Xylene (3) - 3 minutes

### 4.7 Mounting

The stained samples were mounted with Canada balsam for permanent preservation.

## 5. Photography

Histological examination of different organ structures was done by taking photographs using light microscope (U-MDOB, Olympus optical Co. Ltd., Japan). Images of prepared slides of different organs were captured by using the DP2- BSW (Olympus, Japan) software.

## 6. Anatomical examination

Shell length, shell width, shell height, weight (total weight/shell weight) and shell weight in each group were measured. The ratios of shell width (%), shell height (%), shell weight (%) of the juvenile abalones in each group was calculated. Finally, the figures obtained were compared with shell length and the ration of shell weight was compared with that of total weight.

## 7. Histological assessment

The adductor muscle was the most important among all the organs of abalones' histological assessment. In the assessment of adductor muscles, histological changes (swelling, vacuolization, atrophy, loss of muscular fibers) were examined. Moreover, their differentiation conditions were also studied.

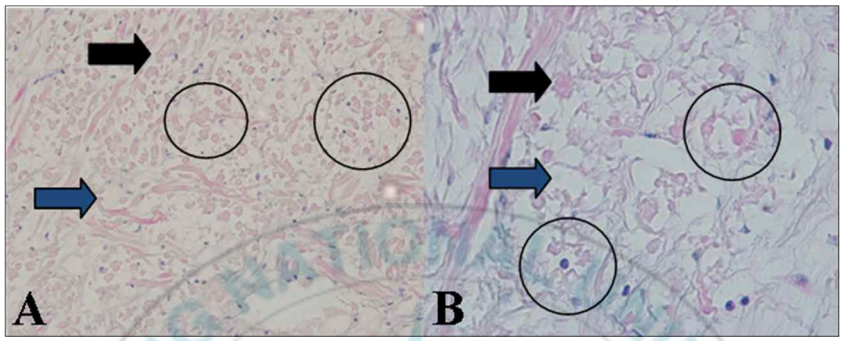


Fig.1. Changes in morphological features of muscle degeneration of adductor muscles. (A: Magnified X400 and B: Magnified X1000.H&E __50 μm).Cellular swelling in adductor muscle fibrils, muscle vacuolization of the adductor muscle fibres (arrows). Cellular atrophy, degenerative muscle fibrils, myonecrosis (elliptical circles) in muscle fibres and loss of muscle fibres in adductor muscle are shown in both figures (1-A, 1-B. H&E __50 μm).

# III. Results

## 1. First sampling (0 weeks)

Before the application of Bio-energy water, juvenile abalone (n=8) were collected for each sampling and the results are as under:

### 1.1 Anatomical measurement

Abalones (*Haliotis discus hannai*) shell length (cm), shell width (cm), shell height (cm), shell weight (g) and total weight (g) of (n=8) was measured during the dissection. Then the ratio of shell height to shell length, ratio of shell width to shell length, ratio of total weight to shell length and ratio of shell weight to total weight were shown in Table 1.

Table 1. Body weight and shell measurement of abalones in first sampling at 0 weeks[1]

| Measurement | Abalones in 0 weeks |
|---|---|
| SL[2] (cm) | 5.86±0.38 |
| Sw[3] (cm) | 3.83±0.29 |
| SH[4] (cm) | 1.14±0.18 |
| Ratio of SH/SL (%) | 19.4±0.1 |
| Ratio of SW/SL (%) | 65.4±1.4 |
| TW[5] (g) | 26.77±2.16 |
| Ratio of TW/SL (%) | 465.6±14.8 |
| SW[6] (g) | 5.13±0.72 |
| Ratio of SW/TW (%) | 19.2±15.3 |

[1]Values are mean±SD (n=8)
[2]SL = Shell Length
[3]SW=Shell Weight
[4]SH=Shell Height
[5]TW= Total Weight
[6]SW=Shell Weight

The abalones in the first sampling were used as an example for the evaluation of abalone adductor muscle health condition. Adductor muscles of 8 individuals were observed. Bioenergy water was used to culture abalone in order to observe post bio-energy water treatment changes in adductor muscle structure. Adductor muscle around the muscle attachment portion was vitrified (hyalinization), vacuolized and the muscle castle was somewhat hollow or swollen because of vacuolization. The findings of this sampling indicated that the whole of muscle was necrotic that had undergone muscle atrophy. Atrophic lesion in the adductor muscle was clearly observed in all 8 individuals. The Epilysium (Perimysium and muscle bundle) had free spaces that indicated declination in the muscle fiber diameter. (Fig.2:1-08). It was observed that the normal structure of the adductor muscle fibres in the juvenile abalone were distorted because of vacuolization. Atrophic lesion of the adductor muscle (muscle fiber loss) was clearly observed in the Epilysium (Perimysium and the muscle bundle) level. The same observations were made in the shell and adductor muscle attachment portions. Generally, there appeared more empty spaces in the adductor muscles and the adductor muscle fibrils. Endomysium, surrounding the muscle, was observed in many cases which did not disappear. The remaining muscle fibres were displaying evident distortions of vacuolization. Outline in the case of a thick muscle fibres showed a concentration in the overall trend, but the range was increasing large in the diameter as shown in (Fig.2:09). All those points described above were observed in the next experiment for the evaluation of histological changes

which were observed after the abalone were cultured in general water and bioenergy water respectively.

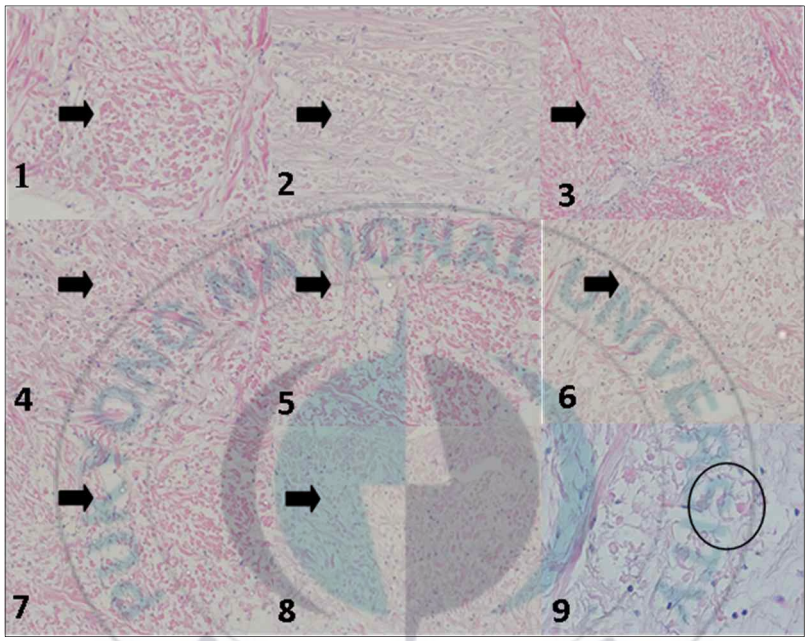


Fig. 2. Photos of adductor muscle of abalone before applying bioenergy water (1-8: 400 times). Muscle fiber lesion of hypertrophy, vacuolization, swelling, and necrosis appear. Vacuolization, lesion, atrophy, and muscle fiber loss (arrows) are clearly visible in images 1-8. In image (9: X1000 magnified) lesion in muscle histology (elliptical circle) is clearly shown (H&E __50 μm).

## 2. Second sample (one week after applying bio-energy water)

### 2.1 Anatomical measurement

Juvenile abalones *(Haliotis discus hannai)* were applied bio-energy water for one week. Moreover, a comparison of adductor muscles of control and experiment groups was made. Shell length (cm), shell width (cm), shell height (cm), shell weight (g) and total weight (g) of (n=8) abalone were measured. Then the ratio of shell height to shell length, ratio of shell width to shell length, ratio of total weight to shell length and ratio of shell weight to total weight were shown in Table 2.

Table 2. Body weight and shell measurement of abalones in second sample at 1 weeks[1]

| Measurement | Abalones in general water | Abalone in Bio-energy water |
|---|---|---|
| SL[2] (cm) | 5.86±0.38 | 4.83±0.08 |
| SW[3](cm) | 3.83±0.29 | 3.32±0.10 |
| SH[4] (cm) | 1.14±0.18 | 0.93±0.08 |
| Ratio of SH/SL (%) | 19.4±0.1 | 19.3±1.7 |
| Ratio of SW/SL (%) | 65.4±1.4 | 68.4±1.2 |
| TW[5](g) | 26.77±2.16 | 60.75±5.09 |
| Ratio of TW/SL (%) | 456.6±14.8 | 1272.5±40.1 |
| SW[6] (g) | 5.13±0.72 | 15.91±1.91 |
| Ratio of SW/TW (%) | 19.2±15.3 | 25.9±7.8 |

[1]Values are mean±SD (n=8)
[2]SL = Shell Length
[3]SW=Shell Weight
[4]SH=Shell Height
[5]TW= Total Weight
[6]SW=Shell Weight

## 2.2 Juveniles' adductor muscle histology evaluation

### 2.2.1 Abalones in general water group

Example photos of the abalone in general water group are shown in Fig.3.Adductor muscle of the juvenile abalone in general water had clear lesion. Vacuolization was clearly observed followed by swelling of the muscle fibers.The diameter of the muscle fiber declined because of atrophy. The muscle was vitrified (hyalinization) and underwent necrosis (Fig 3. 09 enlarged 1000 times).

### 2.2.2 Abalone in bio-energy water group

Example photos of the abalone in bio-energy water group are shown in Fig.4. Histologically there were some clear differences in the muscle structure of the second sample after application of bioenergy water than that of first sample before the application of bio-energy water. The muscle fiber histology showed improvement. The muscle fibers increased in density and grew thicker. A decrease in swelling and muscle fiber vacuolization was observed (Fig 4. 09 enlarge 1000 times).

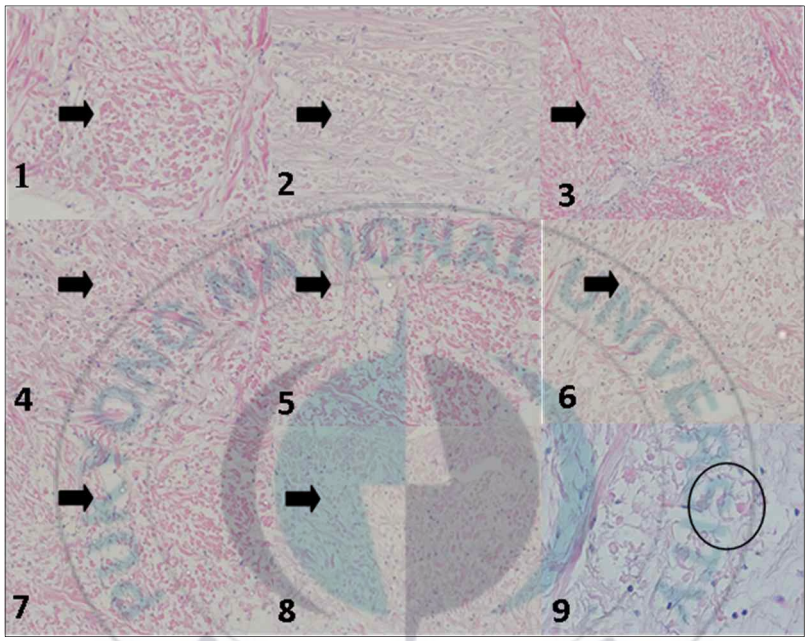


Fig. 3. Photos of the adductor muscle of abalone in general water after one week (1-8: 400 times). Vacuolization, vitrification, swelling, thin diameter, lesion, atrophy and muscle fiber loss (arrows) are clearly visible in images 1-8. In image (9: X1000 magnified) lesion in muscle histology (elliptical circle) is clearly shown (H&E_50 μm)

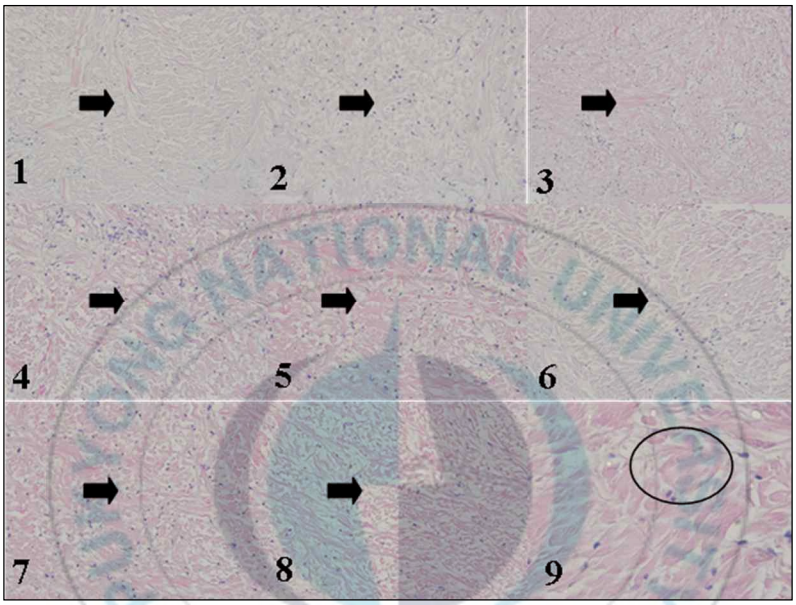


Fig. 4. Photos of the adductor muscle of abalone in bio-energy water after one week (1-8: 400 times). The muscle fiber grew thicker. Vacuolization and swelling gradually decreased (arrows) in images 1-8 as compared to the first sample. In image (9: X1000 magnified) improvement in adductor muscle histology (elliptical circle) is shown (H&E __50 μm).

## 3. Third sampling (two weeks after applying bio-energy water)

### 3.1 Anatomical measurement

Juvenile abalones were cultured for two weeks in bioenergy water. Eight (8) individuals were randomly selected. Shell length (cm), shell width (cm), shell height (cm), shell weight (g) and total weight (g) of (n=8) abalone were measured. Then the ratio of shell height to shell length, ratio of shell width to shell length, ratio of total weight to shell length and ratio of shell weight to total weight were shown in Table 3.

Table 3. Body weight and shell measurement of abalones in third sample at 2 weeks[1]

| Measurement | Abalones in general water | Abalone in Bio-energy water |
|---|---|---|
| SL[2] (cm) | 5.84±0.38 | 4.82±0.13 |
| SW[3](cm) | 3.84±0.29 | 3.35±0.22 |
| SH[4] (cm) | 1.14±0.18 | 0.94±0.10 |
| Ratio of SH/SL (%) | 19.4±0.1 | 19.5±2.7 |
| Ratio of SW/SL(%) | 65.4±1.4 | 69.4±1.3 |
| TW[5](g) | 26.77±2.16 | 16.23±2.69 |
| Ratio of TW/SL (%) | 456.6±14.8 | 69.2±32.3 |
| SW[6] (g) | 5.13±0.72 | 3.33±0.28 |
| Ratio of SW/TW (%) | 19.2±15.3 | 20.6±9.2 |

[1]Values are mean±SD (n=8)
[2]SL = Shell Length
[3]SW=Shell Weight
[4]SH=Shell Height
[5]TW= Total Weight
[6]SW=Shell Weight

## 3.2 Juveniles' adductor muscle histology evaluation

### 3.2.1 Abalone in general water group

Example photos of the abalone in general water group are shown in Fig.5. Adductor muscle of the juvenile abalone in general water had clear lesion. Vacuolization was clearly observed followed by swelling of the muscle fibers. The diameter of the muscle fiber declined because of atrophy. The muscle was vitrified (hyalinization) and underwent necrosis (Fig 5. 09: 1000 times enlarged).

### 3.2.2 Abalone in bio-energy water group

Example photos of the abalone in bio-energy water group are shown in Fig.6. Histologically there was a clear difference observed in the muscle structure of the third sample after application of bioenergy water than that of first and second samples alike. Lesions, swelling of the adductor muscle, vitrification (hyalinization) and vacuolization of the muscles disappeared. The muscle fiber histology showed a clear improvement. The muscle fibers increased in density and grew thicker (Fig 6. 09: 1000 times enlarged). The muscle fiber recovery was observed in all the juvenile abalone cultured in bio-energy water.

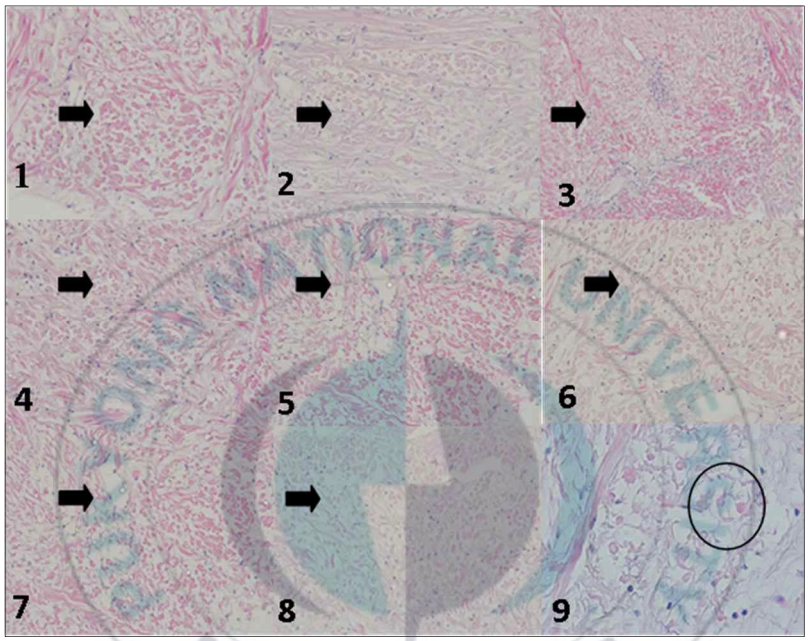


Fig. 5. Photos of adductor muscle of abalone in general water after two weeks (1-8: 400 times). Muscle fiber lesion of hypertrophy, vacuolization, swelling, and necrosis appear. Vacuolization, lesion, atrophy, and muscle fiber loss (arrows) are clearly visible in images 1-8. In image (9: X1000 magnified) lesion in muscle histology (elliptical circle) is clearly shown (H&E __50 μm).

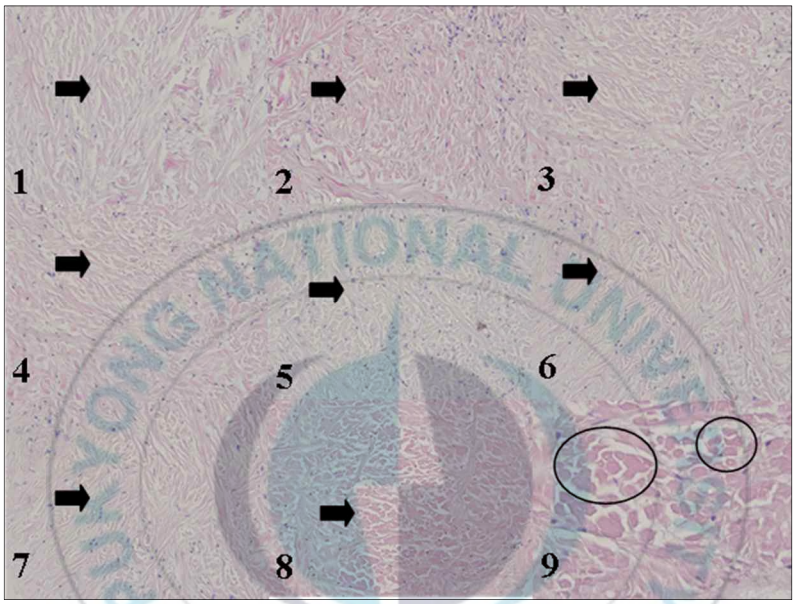


Fig. 6. Photos of adductor muscle of abalone in bio-energy water after two weeks (1-8: 400 times). Lesions, swelling of the adductor muscle, vitrification (hyalinization) and vacuolization of the muscles disappeared (arrows). Muscle fiber thickness and diameter improved as shown in images1-8. In image (9: 1000 times enlarged) adductor muscle recovery (elliptical circles) can clearly be observed (H&E __50 μm).

# 4. Fourth sampling (three weeks after applying bioenergy water)

## 4.1 Anatomical measurement

Juvenile abalones were cultured for three weeks in bioenergy water. Eight (n=8) juvenile abalones were randomly selected from each general water and bio-energy water. Shell length (cm), shell width (cm), shell height (cm), total weight (g) and weight of the shell (g) were measured. Then the ratio of shell height to shell length, ratio of shell width to shell length, ratio of total weight to shell length and ratio of shell weight to total weight were shown in Table 4.

Table 4. Body weight and shell measurement of abalones in fourth sample at 3 weeks[1]

| Measurement | Abalones in general water | Abalone in bio-energy water |
|---|---|---|
| SL[2] (cm) | 5.86±0.38 | 5.06±0.34 |
| SW[3](cm) | 3.83±0.29 | 3.85±0.56 |
| SH[4] (cm) | 1.14±0.18 | 1.15±0.20 |
| Ratio of SH/SL (%) | 19.4±0.14 | 22.8±2.7 |
| Ratio of SW/SL (%) | 65.4±1.4 | 76.2±10.2 |
| TW[5] (g) | 26.77±2.16 | 66.68±11.51 |
| Ratio of TW/SL (%) | 456.6±14.8 | 1316.6±43.7 |
| SW[6](g) | 5.13±0.72 | 18.32±1.90 |
| Ratio of SW/TW (%) | 19.2±15.3 | 27.5±34.2 |

[1]Values are mean±SD (n=8)
[2]SL = Shell Length
[3]SW=Shell Weight
[4]SH=Shell Height
[5]TW= Total Weight
[6]SW=Shell Weight

## 4.2 Juveniles' adductor muscle histology evaluation

### 4.2.1 Abalone in general water group

Example photos of the abalone in general water group are shown in Fig.7. Adductor muscle of the juvenile abalone in general water had clear lesion. Vacuolization was clearly observed followed by swelling of the muscle fibers. The diameter of the muscle fiber declined because of atrophy. The muscle was vitrified (hyalinization) and underwent necrosis. The adductor muscle had no better condition. Muscle fiber along the shell portion showed (hypertrophy), vacuolization, atrophy, necrosis, and thick Epilysium diameter (Fig 7. 09: 1000 times enlarged).

### 4.2.2 Abalone in bio-energy group

Example photos of the abalone in bio-energy water group are shown in Fig.8. Juvenile abalone cultured in bio-energy water group showed improvement. Shell mounting portion of the adductor muscle, vitrification (hyalinization), vacuoles in cytoplasm (vacuolization), atrophy and muscle fiber loss improved significantly. A clear improvement in the muscle density was observed. Swelling and vacuolization in the muscle fiber almost disappeared in most of the abalones.

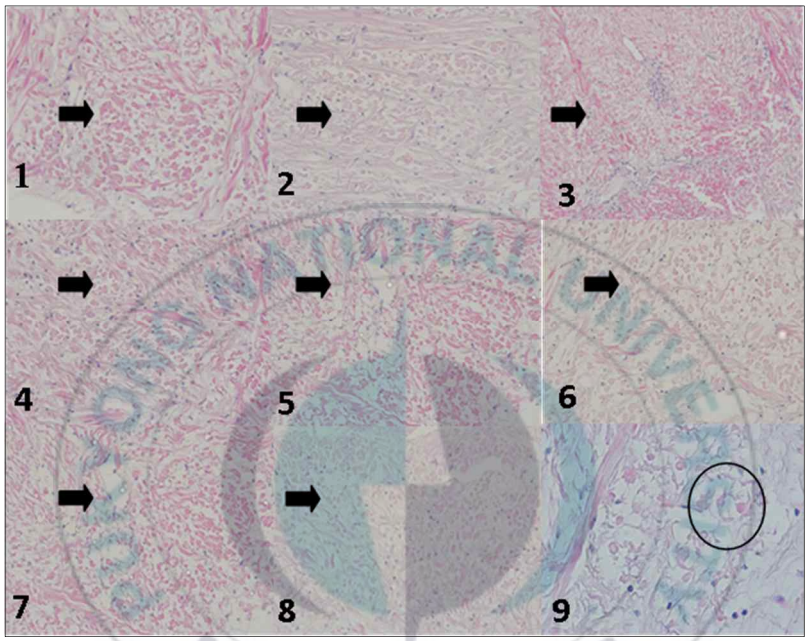


Fig. 7. Photos of adductor muscle of abalone in general water after three weeks (1-8: 400 times).Vacuolization, lesion, hypertrophy, atrophy and muscle fiber loss (arrows) are clearly visible in images 1-8. In image (9: X1000 magnified) lesion in muscle histology (elliptical circle) is clearly shown (H&E __50 μm).

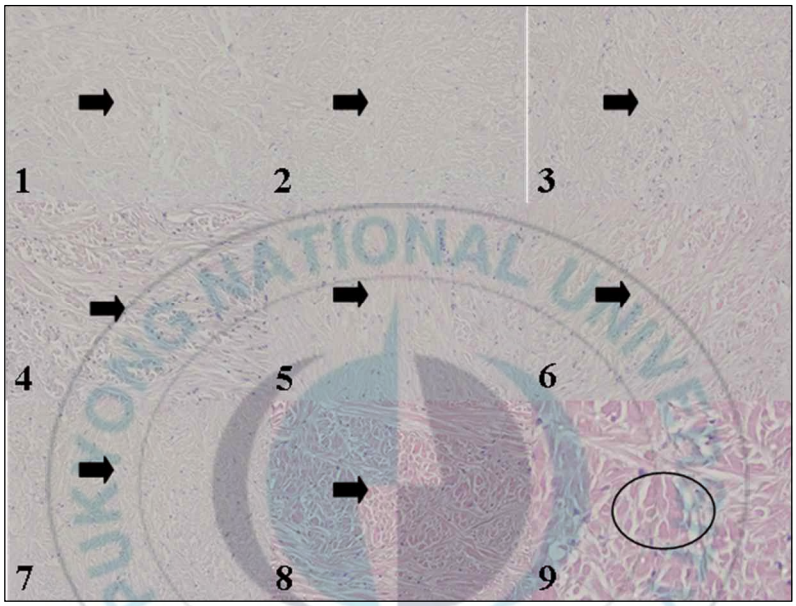


Fig. 8. Photos of adductor muscle of abalone in bio-energy water after three weeks (1-8: 400 times).Shell mounting portion of the adductor muscle, vitrification (hyalinization), vacuoles in cytoplasm , atrophy and muscle fiber loss improved significantly (arrows). Swelling and vacuolization in the muscle fiber almost disappeared. In image (9: 1000 times enlarged) muscle lesion (elliptical circle) also disappeared (H&E __50 μm).

# 5. Fifth sampling (four weeks after applying bioenergy water)

## 5.1 Anatomical measurement

Juvenile abalones were cultured for four weeks in bioenergy water. Eight (n=8) juvenile abalones were randomly selected from each general water and bio-energy water. Shell length (cm), shell width (cm), shell height (cm), total weight (g) and shell weight (g) were measured. Then the ratio of shell height to shell length, ratio of shell width to shell length, ratio of total weight to shell length and ratio of shell weight to total weight were shown in Table 5.

Table 5. Body weight and shell measurement of abalones in fifth sample at 5 weeks[1]

| Measurement | Abalones in general water | Abalone in bio-energy water |
|---|---|---|
| SL[2] (cm) | 5.85±0.38 | 4.93±0.40 |
| SW[3](cm) | 3.84±0.29 | 3.38±0.23 |
| SH[4] (cm) | 1.15±0.18 | 0.98±0.12 |
| Ratio of SH/SL (%) | 19.4±0.1 | 20.2±2.8 |
| Ratio of SW/SL (%) | 65.4±1.4 | 68.6±1.7 |
| TW[5] (g) | 26.77±2.16 | 61.31±9.42 |
| Ratio of TW/SL (%) | 456.6±14.8 | 1241.4±42.7 |
| SW[6](g) | 5.14±0.72 | 16.84±3.02 |
| Ratio of SW/TW (%) | 19.2±15.3 | 27.4±31.4 |

[1]Values are mean±SD (n=8)
[2]SL = Shell Length
[3]SW=Shell Weight
[4]SH=Shell Height
[5]TW= Total Weight
[6]SW=Shell Weight

## 5.2 Juveniles' adductor muscle histology evaluation

### 5.2.1 Abalone in general water group

Example photos of the abalone in general water group are shown in Fig.9. Adductor muscle of the juvenile abalone in general water had clear lesion. Vacuolization was clearly observed followed by swelling of the muscle fibers.The diameter of the muscle fiber declined because of atrophy. The muscle was vitrified (hyalinization) and underwent necrosis. Muscle fiber along the shell portion showed (hypertrophy), vacuolization, atrophy, necrosis, and thick Epilysium diameter (Fig 9. 09: 1000 times enlarged).

### 5.2.2 Abalone in bio-energy group

Example photos of the abalone in bio-energy water group are shown in Fig.10. Juvenile abalone cultured in bio-energy water group for four weeks showed great improvement. Recovery in the histology of the muscle fibers and the muscular bundle was observed in majority of the individuals of this sample (n=8). The improvements observed in the adductor muscle were much clearer in most of the cases. Muscle degeneration also disappeared in many of the cultured abalones (Fig 10.09: 1000 times enlarged).

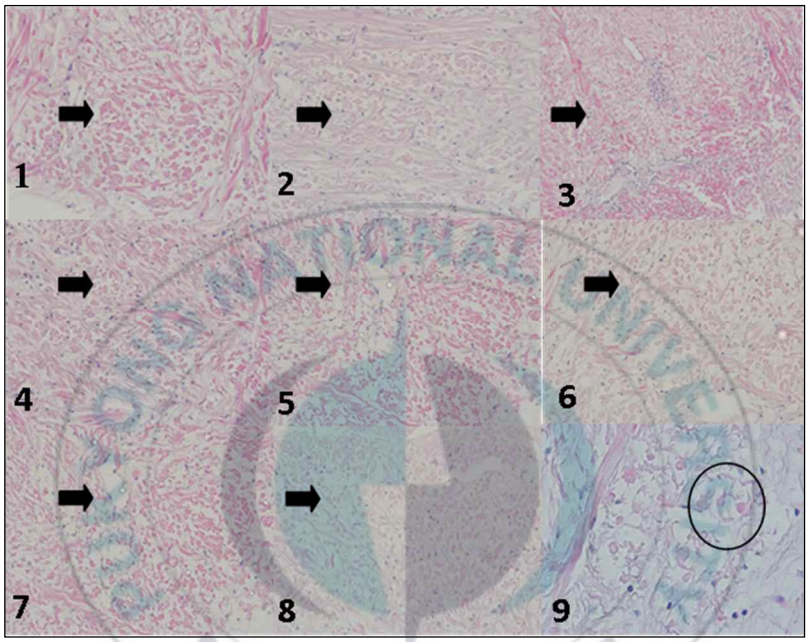


Fig. 9. Photos of adductor muscle of abalone in general water after four weeks (1-8: 400 times).Vacuolization, lesion, hypertrophy, atrophy and muscle fiber loss (arrows) are clearly visible in images 1-8. In image (9: X1000 magnified) lesion in muscle histology (elliptical circle) is clearly shown (H&E __50 μm).

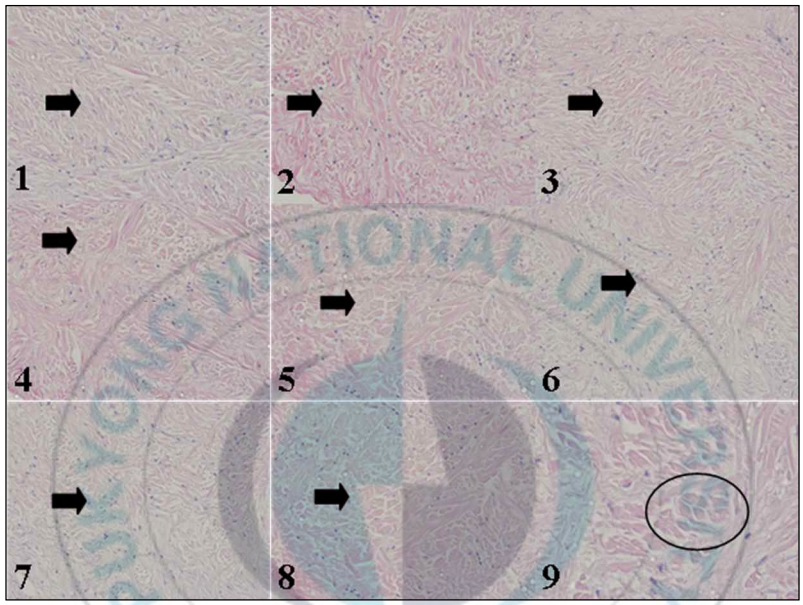


Fig. 10. Photos of adductor muscle of abalone in bio-energy water after four weeks (1-8: 400 times).The muscle fibers and the muscular bundle recovered in all individuals of this sample (n=8). Increase in muscle fiber diameter, no vacuolization or swelling of the muscle fibers (arrows) is clearly shown. In image (9: 1000 times enlarged) the complete adductor muscle fiber recovery (elliptical circle) is visible (H&E __50 μm).

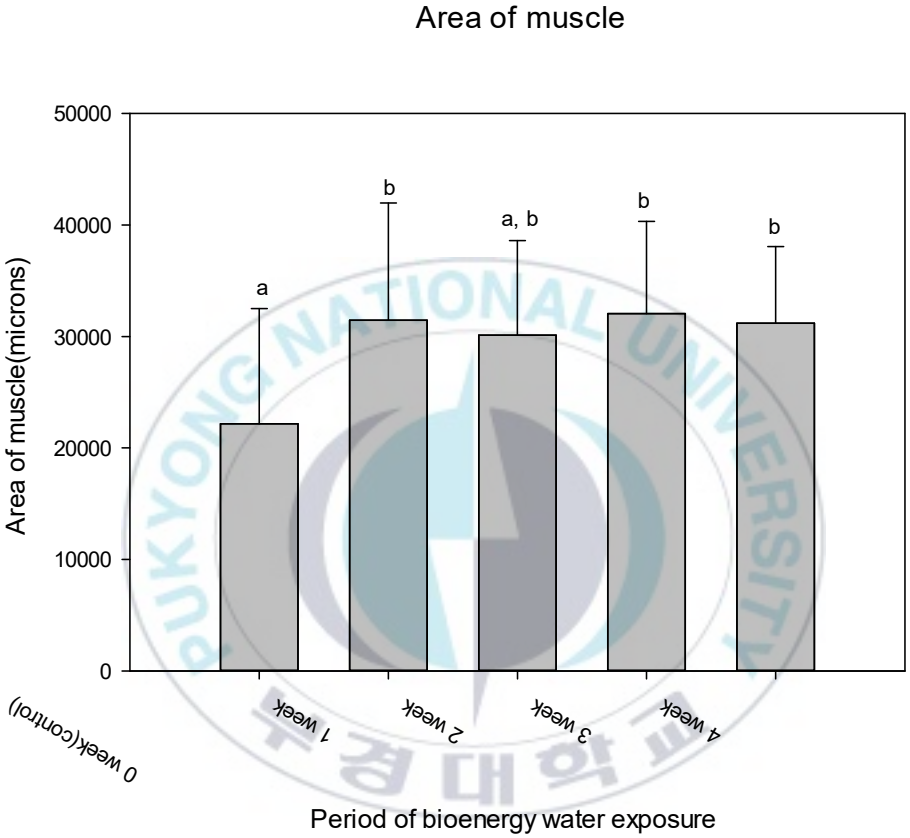


Fig. 11. Adductor muscle recovery in juvenile abalone in control group (general water, 0 weeks) compared with that of treatment groups (bio-energy water, 1-4 weeks).The area of muscle in adductor muscle was calculated with 400 magnification photograph.

# IV. DISCUSSION

The prime objective of this study was to assess the recovering effects of bio-energy water on the lesion found in the degenerated adductor muscles of juvenile abalone *(Haliotis disucs hannai).* Bio-energy water, used in this study, was provided by DM Bio Company Limited, Korea. The adductor muscle lesions of abalone, cultured in bio-energy water, were examined under light microscope in order to examine the recovering process of adductor muscle lesion after being cultured in bioenergy water.

The results were remarkably positive. Abalone in the first sample (before the application of bioenergy water) exhibited complete necrosis. Lesion was found distributed in the portion of adductor muscles which is normally attached to the outer shell area of the animal body. Muscle fiber degeneration, vacuolization, hyalinization and muscle swelling were caused by necrosis. In the second sample (1 week after the application of bioenergy water) there was a great improvement in the adductor muscle histology. In the third sample (2 weeks after the application of bioenergy water) muscle fibers increased in density and started to grow thicker after being cultured in bioenergy water with complete recovery in almost all individuals. In fourth sample (3 weeks after the application of bioenergy water) lesion, swelling of the adductor muscle, vitrification (hyalinization) and vacuolization of the muscles disappeared gradually with a clear improvement in muscle

fiber histology in most of the abalones. In fifth and final sample (4 weeks after the application of bioenergy water), shell mounting portion of the adductor muscle fiber improved significantly. Vitrification (hyalinization), vacuoles in cytoplasm (vacuolization), cellular atrophy reduced and muscle fibrils grew thicker in diameter. Muscles were observed with no swelling and vacuolization in the muscle fiber in most of the individuals. These all changes occurred because of bioenergy water and its efficacy in muscle lesion recovery.

Not much work has so far been done, except a few, on the immunopotentiating characteristics and adductor muscle repairing properties of bioenergy water. Adductor muscle fibrils in adult abalones recovered in the third week after being cultured in bioenergy water for two months in fasting condition (Thearith Em 2015). Adductor muscle fibrils did not recover in any of the juvenile abalones cultured in bioenergy water for three months in excessive feeding condition (Chankakada Mak, 2016). This might have been because of free radicals and super oxide dismutase (SOD) produced due to excessive feeding. Therefore, this study was conducted on juvenile abalone cultured in bioenergy water in fasting condition so as to avoid any negative effects of overfeeding, free radicals, food toxicants etc., on the structural changes of adductor muscles after being cultured in bioenergy water.

Adductor muscle is an important organ of the abalone body as it is attached with the shell of the animal body. It maintains the balance and movement of the whole of abalone body. It also helps abalone attach to the substratum (Fretter & Graham 1962). Any problems in

the adductor muscles (lesion, necrosis) causes the detachment of adductor muscle from the main body, which in return, causes mortality. Muscle degeneration in abalone due to high temperature causes mass mortality. It also causes detachment of muscle fibrils from the main shell that leads to death (Bo Seong Kim et al., 2014, Dang VT et al., 2012, Kim Th et al., 2005).But the real mechanism of muscle necrosis or complete degeneration has not been understood.

Some studies have also reported that SOD (superoxide dismutase), hydrogen peroxide ($H_2O_2$), oxidative stress, accumulation of toxic ammonia may also cause muscular necrosis or muscle degeneration in abalones primarily in culture farms where abalones are fed with excessive food for the purpose of their fast growth. Over feeding produces the aforementioned lethal materials injurious to abalones' life (Zou et al., 2013, Guenther et al, 2015).

As stated earlier, the main objective of this study was to assess the efficacy of bioenergy water in recovering degenerated adductor muscles of juvenile abalone. Bio-energy water is devoid of any chemical additives. This special type of water in obtained by passing solar energy (life-giving energy having 4-14 μm wave length) through common water. Bioenergy water ensures maximum absorbance and bioavailability of water molecule in the body.

Our results in this study proved the efficacy of bioenergy water. Degenerated adductor muscles (necrotic muscles) showed remarkable recovery in juvenile abalone reared in bioenergy water for four weeks. The results obtained in this study (under fasting

condition) are similar to the results achieved in case of adult abalone cultured in bioenergy water (under fasting condition for three months ) reported by (Thearith2015).

Water is the matrix of life. Water, a polar molecule made up of two electro positively charged hydrogen atoms and an electronegative oxygen atom, is a simple molecule but possesses amazing properties. All the physiological activities in the living organisms depend upon water. Animal body is composed of numerous macro and micro molecules which comprise 30% of the total body, whereas water constitutes the remaining 70% of the body (Martin.F.Chaplin, 2001). Cell structure, its functions inside and outside environments are maintained by water. Hydrogen bond (water) plays a pivotal role in the structural formation of biomolecules such as DNA, RNA, proteins, fats, carbohydrates etcetera. All the biochemical activities of the cell are performed by water (Martin.F .Chaplin, 2006).

Water is the reality of immunity. Immunity, which is the power of a living organism to remain alive, is based on the structure-function relationship. Any disturbance in the structure perturbs function or immunity. This maintenance of normal structure –function relationship (or in other words immunity) in the living organisms is performed by water. For example, a flaccid plant, because of dehydration, is reanimated as soon as it is provided with water. This is because dehydration deteriorated the whole of structure-function relationship within plants cell. The same plant is enlivened when it is watered because water reinstates the structure-function relationship, which in return, helps plant

regain its normal structure and function. Thus, it can be said that water (hydrogen bond) is the life giving medium in the living cells.

This innovative idea regarding the role of water in strengthening immune power has still not been known because of prejudice about the role of water. This is because water is considered merely a solvent. It is the unique property of polarity in the water molecule that enables it to make clusters with molecules of its own and connect with other molecules within the cell to sustain all the vital activities of the cell and living organisms. It is high time we understood logically the real role of water, that is, immune strengthening medium, in the living cells and spread this concept across the world.

The results of this study propose that water, the most studied liquid on earth, should no more be underestimated. The recovery of necrotic and degenerated muscles of juvenile abalone used in this study as an experiment animal was only possible because of the unique characteristics of water molecule which sustained the original position of muscle fibers, cells and tissues (life giving property of water molecule). Water quality parameters, environmental pollution, toxicants and pathongs have only been held responsible for mass mortality in abalones. This study does not deny the aforesaid causes of mortality in abalone, but suggests, at the same time, that the real role of water should also not be neglected altogether. Keeping in view the health enhancing properties of bioenergy water, it is suggested that bioenergy water can be used for the health improvement purposes in fish and shell fish, since water is the backbone of immunity and structure-function reinstatement in the living organisms. Positive results regarding the application of

bioenergy water in agriculture and livestock industries have already been reported in Korea and Vietnam. Therefore, it is suggested that, both, fish and shell fish be cultured in bio-energy water with moderate feeding (as overfeeding is injurious to fish and shell fish health). This would be cost effective and it would also minimize the risks of lethal diseases (mainly caused by food toxicants) in aquaculture industry.

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# VI. Reference


Andrea, J.M. and A. Katharina.2015. Effects of environmental and farmed stress on abalone physiology. Journal of Aquaculture (Reviews in aquaculture). (2015) 7, 1–27.

Barnes, R.D. 1986. Invertebrate zoology. 5th edn. Saunders: New York. 893 pp.

Boden, G., C.Homko, C.A.Barrero, T.P.Stein, X.H. Chen, P.Cheung, C.Fecchio, S.Koller, S.Merali. 2015. Excessive caloric intake acutely causes oxidative stress, GLUT4 carbonylation, and insulin resistance in healthy men. Sci Trans Med, 2015 Sep 9; 7(304):304re7. Doi: 10.1126/scitranslmed.aac4765.

Burke, C.M., J.O.Haris,S.M.Hindrum,S.J.Edwards and G.B.Maguire.2001.Environmental requirements of abalone. Tasmanian aquaculture and fisheries institute, fisheries research and development corporation, Australian, project number 97/323.

Chaplin, M.F. 2001. Water, its importance to life. Journal of Biochemistry and Molecular Biology Education, 29 (2001), 54-59.

Chaplin, M.F. 2006. Do we understand the importance of water in cell biology? Journal of molecular cell biology volume 7 (2006), 861-866.

Chaplin, M.F. and G. Pollack. 2006. Information exchange within intracellular water. Journal of Water and the cell 2006, volume 7 (2006), 113-123. Chaplin, M.F.2001.

Water, its importance to life. Journal of Biochemistry and Molecular Biology Education, 29 (2001), 54-59.

Chaplin, M.F. 2006. Do we understand the importance of water in cell biology? Journal of molecular cell biology volume 7 (2006), 861-866.

Chaplin, M.F. and G. Pollack. 2006. Information exchange within intracellular water. Journal of Water and the cell 2006, volume 7 (2006), 113-123.

Cheng, W., I.S.Hsiao, J.C.Chen. 2004. Effects of ammonia on the immune response of Taiwan abalone, *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. Fish & Shellfish Immunology 17: 193–202.

Cheng, W., I.S.Hsiao and J.C.Chen. 2004. Effects of Ammonia on the immune responses of Taiwan abalone, *Haliotis diversicolor supertexta,* and its susceptibility to Vibrio parahaemolyticus. Journal of fish and shellfish immunology 17 (2004), 193-202.

Chen, H., K.Mai, W.Zhang, Z.Liufu. 2005. Effects of dietary Pyridoxine on immune responses in abalone, *Haliotis discus hannai*. Fish and Shellfish Immunology, 19, 241–252.

Clark, S.A., A.C.Miller and W.F.Ponder. 2003. Revision of the Snail Genus Austropyrgus (Gastropoda: Hydrobiidae): A Morphostatic Radiation of

Freshwater Gastropods in Southeastern Australia. Records of the Australian Museum, supplement 28(2003): 1–109 ISBN 0-7347-2313-X, p1-5 of 11.

Cox, L.R.1960. Family Haliotidae Rafinesque. 1915. Treatise on invertebrate palaeontology, edited by E. Moore. (I) Mollusca 1, p. 221–233. Lawrence, Univ. of Kansas Press, 351 p.

Cook, P.A. 2016. The world abalone industry. Journal of Modern Economy 5, 1181-1186.

Dang, V.T., P. Speck and K .Benkedorff. 2012. Influence of elevated temperature on the immune response of abalone, *Haliotis rubra*. Fish & shellfish immunology 32, 732-740.

Dang, V.H., P. Speak and K. Benkendoff. 2012. Influence of elevated temperature on the antiviral and antibacterial immune responses of abalone, *Haliotis rubra.* Journal of fish and shellfish immunology, vol. 32, no, pp. 732-740.

Daniel,L.T., L.Geigerand, L.T.Groves.1999.Review of fossil (Gastropoda:Vetigastropoda (Haliotidae) with comparison to recent species. Journal of Palaeontology, 75,51999,872-885

Davidson, R., A.Lauritzen and S.Seneff. 2013. Biological water dynamics and entropy: A biophysical origin of cancer and other disease. Journal of Entropy 15 (2013), 3822-3876.

Ding, J., L.Li, F.Wu and G. Zhang. 2016. Effects of chronic temperature exposure on the immunity of abalone, *Haliotis discus hannai*. Journal of Aquaculture research 47, 2861-2873.

Donovan, D.A. and H.H. Taylor. 2008. Metabolic consequences of living in a wave swept environment: Effects of simulated wave forces on oxygen consumption, heart rate and activity of shell adductor muscles of Abalone, *Haliotis iris*. Journal of Experimental Marine Biology and Ecology, 354 (2), 231-240.

Em, T.H. 2016. The histological effects of bio-energy water on degenerated adductor muscle found endemically in cultured abalone, Haliotis discus hannai. M.S.Thesis, Pukyoung National University, Busan, Republic of Korea.

Evelyne, B. 2000. Introduction of Abalone Immunity and Disease Control. Journal of Aquaculture, (19) 13–11.

F.A.O.1990.Training Manual on Artificial Breeding of Abalone, *Haliotis discus hannai,* in Korea DPR. FAO Demonstration Project (RAS/90/002), Training Manual 7.

F.A.O. 2016.The state of world fisheries and aquaculture .F.A.O. aquaculture production report, 978-92-5-109185-2, 2016.

Fisher, W.S., R.I.E.Newell.1986. Salinity effects on the activity of granular haemocytes of American oysters, *Crassostrea virginica*. Biological Bulletin 170: 122–134.

Fretter, V. and A.Graham.1962. British Prosobranch Molluscs, their functional anatomy and ecology, 755 pp. Ray Society, Bartholomew Press, Dorking.

Frescura, M. 1990. Aspects of the structure and function of some gastropod columellar muscles (Mollusca). Thesis submitted in fulfilment of the requirements for the degree Doctor of Philosophy of Rhodes University, 175 pp.

Geiger, D.L. 1999. Distribution and biogeography of the recent Haliotidae (Gastropoda: Vetigastropoda) world-wide. Bollettino Malacologico 35:57-120.

Gorfine, H.K., R. Tailby, F.Gant, M.Donaldson, I. Bruce. 2002. Estimation of illegal catches of Australian abalone: Development of desk-based survey methods. F.R.D.C. Project 2000/112 final report. Marine and Freshwater Resources Institute: Queens' cliff, Victoria, Australia.

Gorfine, H.K. 2002. Assessment of the sustainability of Victorian abalone resources .A thesis submitted in total of the requirements of Degree of Doctor of Philosophy, University of Technology, Sydney, Australia.

Hahn, K.O. 1989c. Survey of the commercially important abalone species in the world. Handbook of culture of abalone and other marine gastropods. CRC Press: Florida, Pp. 311-15.

Harris, J.O., G.B.Maguire, S.J.Edwards, D.R.Johns. 1999a. Low dissolved oxygen reduces growth rate and oxygen consumption rate of juvenile green lip abalone, *Haliotis laevigata donovani*. Journal of Aquaculture 174: 265–278.

Hermien, N.C.W. 1995. Anaesthesia in abalone, *Haliotis midae* .A thesis submitted in fulfillment of Degree of Master of Science, Rhodes University, South Africa.

Hooper, C., R.Day, R.Slocombe, J.Haldlinger, K.Benkedorff 2016. Stress and immune response in abalone. Limitations in the current knowledge and investigative methods based on other models. Journal of Fish & Shellfish Immunology 22(2007), 363-379.

Huchette, S.M.H., C.S. Koh, R.W. Day. 2003b. Growth of juvenile blacklip abalone, *Haliotis rubra,* in aquaculture tanks: effects of density and ammonia. Journal of Aquaculture 219: 457–470.

Izawa, T., H.Nagai, T.Endo and S.I.Nishikawa. 2012. Yos9p and Hrd1p mediate ER retention of misfolded proteins for ER-associated degeneration. Journal of Molecular Cell biology 23(7):1283-1293.

Kanaujia, S.P and K.Sekar. 2008. Structural and functional role of water molecule in bovine pancreatic phospholipase A2: a data-mining approach. Acta Crystallographica, section, Biological Crystallography, ISSN 0907-4449 D2009, D65, 74-84p.

Keith, W.C. 1992.Californian abalones, Family Haliotidae. Marine Resources Operations Agency of California, document number 118, Pages 6-18.

Kim, T.H., Y.M.Hyu, C.M. Kyung, H.S. Jung, and Y.M.Hyu. 2005. Physiological studies on acute water-temperature stress of juvenile abalone, *Haliotis discus hannai.* J Aquaculture 18, 7-12.

Kim, B.S., S.J.Yi, S.M.Hwang, M.K. Lee, Y.J. Park, K.J. Song and M.D. Huh. 2014. Myopathy in clinically healthy cultured abalone, *Haliotis discus hannai*. Korean Journal of Fish Aquatic Science, 47 (2), 174-178.

Krecara, I.M., M.Kolega, S.F.Kunaca. 2014. Effects of chronic drinking water on attention. Journal of Social and Behavioural Sciences 159(2014), 577-583.

Lee, D.C., Y.C. Park, C.Y. Jeon, J.Y. Yang, Y.B. Hur, J.W. Kim and K.C. Cho. 2013. A report on the 2012 summer mass mortalities of black rockfish, *Sebastes schlegeli* in the Southeast Sea, Korea. Journal of Fish Pathology 26(3):173–183.DOI: 10.7847/jfp.2013.26.3.173.

Lindberg, D.R. 1992. Evolution, distribution and systematics of Haliotidae. In: Abalone of the World. Biology, fishery and culture, eds S.A. Shepard, M.J. Tegner, Guzmán del Próo (eds). Fishing News Books, Wiley-Blackwell, New Jersey, pp. 3-18. S.A.

Mak, C.K.D. 2017. The histological effects of bio-energy water on degenerated adductor muscle of juvenile abalone, *Haliotis discus hannai*. M.S.Thesis, Pukyoung National University, Busan, Republic of Korea.

Mgaya, Y.D., J.P. Mercer. 1995. The effects of size grading and stocking density on growth performance of juvenile abalone, *Haliotis tuberculata Linnaeus*. Aquaculture 136: 297–312.

Nedecky, B.R., L.Nejdl, J. Gumulec, O.Zitka, M.Masarik, T.Eckschlager, M. Stiborova, V.Adam and R.Kizek.2013.Role of Metallothionein in oxidative stress. International Journal of Molecular Science, 14(3), 6044-6066.

Nie, Z.Q. 1992. A review of abalone culture in China. P.592-602.In: Abalone of the world. Biology, fisheries and culture. S.A. Shepherd, M.J. Tegner & S.A. Guzman del Pr6o (eds.). Blackwell: Oxford, U.K.

Park, C.J., B.H.Min, K.S. Kim, J.H. Lee, J.K. Noh, H.C. Kim, J.W. Park and J.I. Myeong. 2011. Physiological responses on low water-temperature stress of pacific abalone, *Haliotis discuss Hannai.* Korea J Malacology 27, 317-322.

Robinson, G., N.McGowan, P.Leighton. 2008. Abalone hatchery manual. Aquaculture technical section report, 25. Aquaculture Development Board, Dublin, Ireland.

Shumway,S.E. 1977.Effects of salinity fluctuation on the osmotic pressure and Na+ , Ca2+ and Mg2+ ion concentrations in the haemolymph of bivalve molluscs. Journal of Marine Biology 41: 153–177.

Trueman, E.R and A.C.Brown. 1985. The mechanism of shel1 elevation in Haliotis (Mollusca: Gastropoda) and a consideration of the evolution of the hydrostatic skeleton in Mollusca. 1. Zool., Lond, 205: 585-594.

Voltzow, J. 1990. The functional morphology of the pedal musculature of marine gastropods *Busycon contrarium* and *Haliotis kamtschatkana*. The Veliger, 33(1): 1-19 (January 2, 1990).

Watterson, J.G. 1987. A role for water in cell structure. Biochemical Journal. 248(2), 615-617.

Zou, L., A.Shiah, W.J.Roberts, M.T. Chien, P.D. Wagner, M.C. Hogan. 2013. Low Po (2) conditions Induces reactive oxygen species formation during contraction in single skeletal muscles fibers. Am J Physiol Regul Integr Physiol 2013, June 1; 304(11):R1009-R1016.