Thesis for the Degree of Master of Fisheries Science

Isolation and characterization of microalgae from the digestive system of hard clam *(Meretrix lusoria)*

by

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대합 소화기관으로부터의 미세조류 분리 및 특성 연구 *(Meretrix lusoria)*

Advisor: Prof. Choi Tae Jin

by

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Abstract

A total of 34 microalgae species were isolated from the digestive tract of hard clam (*Meretrix lusoria*). Among them, 32 species were identified by microscopic observation and comparison with reference pictures. Two unidentified microalgae names U4 and U5 were further characterized. The 18S rRNA genes were successfully amplified by common 18S rRNA gene primers from these isolates. Sequence analysis of these amplified DNA form the solate U4 and U5 showed 97% and 98% sequence similarity to those of *Neorhodella cyanea* and *Minutocellus polymorphus*, respectively. Scanning electron microscopic observation also confirmed the species identification. Both *Neorhodella* sp. and *Minutocellus* sp. showed the highest growth at 18 ^{o}C with the specific growth rate and number of divisions per day of 1.62±0.39 and 2.24, and 0.39±0.01 and 0.56, respectively.

Key words: microalgae, hard clam, *Meretrix lusoria*, *Neorhodella*, *Minutocellus.*

Introduction

Hard clam, *Meretrix lusoria*, is an abundant bivalve in East and Southeast Asia (Yoosukh & Matsukuma, 2001). It is normally found in estuaries and intertidal zones where salinities are between 20‰ and 33‰ and is a benthic organism which always remains at sites between 5 and 10 cm in depth under aerobic conditions (Lee et al., 2007).

Cultivation of this clam has been growing and has served as an important shellfish distributed around Japan, Korea and China (Chung, 2007). According to Karnjanapratum et al. (2013), *Meretrix lusoria* were rich in polyunsaturated fatty acids (46.84−49.18% of total fatty acid) with high level of DHA (13.33−16.47 % of total fatty acids) and EPA (4.75−7.11% of total fatty acids) and macro- (Na, K, Ca and Mg) and micro- (Fe, Zn, Cu and Cr) minerals. Therefore, this clam is an excellent nutritive source, which could be beneficial for the health of the consumers.

In addition, *Meretrix lusoria* is a popular seafood and traditionally used as a Chinese remedy for liver disease and chronic hepatitis. Research

indicates that the induction of apoptosis may prove to be a pivotal mechanism for its cancer chemopreventive action (Pan et al., 2006; 2007).

Microalgae play an important role in the marine ecosystem as a primary producer which contribute to maintain the biological production and eventually to enhance the fishery production, especially for larval and juvenile bivalves, and for the larvae of some crustacean and fish species in mariculture. Microalgae used for larval nutrition usually are in the nanoplankton size range (2-20μm) (Brown et al., 1997). Intensive rearing of bivalves has so far relied on the production of live algae, which generally accounts for about 30% of the total seed production cost (Coutteau and Sorgeloos, 1992). Previous studies on development of microalgal strains for aquaculture hatchery production have been done for; blue mussel *Mytilus galloprovincialis* (Pettersen et al., 2010), Pacific oysters *Crassostrea gigas* (Knuckey et al., 2002; Barille´ et al., 2003), Greenshell™ mussel *Perna canaliculus* (Ragg et al., 2010), European oyster *Ostrea edulis* (Ronquillo et al., 2012), abalone *Haliotis diversicolor* (Chen, 2007).

Microalgae are usually classified primarily based on morphological characteristics, and size through the use of a light microscope. However, other smaller microalgae (pico-size cells) have remained elusive because

they lack morphological features for identification; sometimes, they are even ignored in cell counting by microscopy. This is especially true for Coccoid organisms, often referred to as “little green balls” or “little round green things”; they are extremely difficult to classify, because of their small size (often <5 μm) and simple morphologies (Soylu and Gönülol, 2012). Thus, molecular technologies have greatly expanded our understanding of the diversity of microalgae that may not be detected by microscopy. For many types of microorganisms, the gene most commonly employed for diversity studies is the small-subunit ribosomal RNA gene (16S rRNA in prokaryotes and 18S rRNA in eukaryotes). Alternatively, metagenomic analysis has been applied to study the microalgal diversity, by comparing the 18S rRNA sequences as DNA taxonomic markers, because significant sequence data are available in public databases, such as GenBank. However, not all microalgae have been sequenced, despite the obvious bias that recovered sequences may represent known species. The magnitude of novel sequences, even in well-known lineages, has revealed many cryptic species (Sarno et al. 2005). Molecular techniques are now used in many areas and have increased the confidence in studies in other countries.

Therefore, the aim of this study was to determine the composition of microalgae in the digestive tract of hard clam (*Meretrix lusoria*) and to identify the isolated microalgae based on molecular and morphological characteristics.

Materials and Methods

Classification and isolation of microalgae

Hard clam were collected from the Gomso Port, Buan-gun, Jeollabuk, Korea in October 2014.

Microalgae from the digestive tract of hard clam were identified based on morphological features using a light microscope with digital camera. Taxonomic identifications were performed according to the methods of Tomas, C. R., (1997); Presscott, G. W., (1951).

Microalgae species were isolated in the laboratory using an inverted microscope and the Pasteur pipette method (Hoshaw and Rosowski, 1973). Isolated microalgae were grown and produced in modified f/2 medium (Guillard and Ryther, 1962; Fritz Industries, Inc. 1997) at 18 ^{o}C under 12:12h light/dark cycle with cool white fluorescent lighting of 90 μmol photons.

To obtain axenic monospecies, cultured microalgae were checked daily under an inverted microscope and streaked on LB agar plate to check for

bacteria prescence. In case the cultured microalgae solutions still contained mixed other species, then the isolating protocols were repeated until monospecific cultures emerged. And incase LB plates had bacterial colonies, cultured microalgae were treated with Spenicillin 100μg/mL then checked on LB plates to ensure no bacterial colonies.

Molecular identification of unknown microalgae

In order to further identify the strain species, the genomic DNA was extracted based on the Brief Protocol of GeneAll® DNA purification system (GeneAll Biotechnology Co., LTD). PCR were performed in a final volume of 20 μL containing 0.5 μL of each primer, 1 μL genomic DNA, 8.5μL D.W. and 1×Prime Taq Premix (GENET BIO, Korea). Two universal eukaryotes primers, 18SF (forward, 5′-CCTGGTTGATCCTGCCAG-3′) and 18SR (reverse, 5′-TTGATCCTTCTGCAGGTTCA-3′), were used for PCR amplification of the 18S rDNA. The PCR reaction was performed with a thermal program, which consisted of preheating at 95 °C for 2 min, 36 cycles of denaturation at 94 °C for 20 sec, annealing at 55 °C for 10 sec, and

extension at 72 ℃ for 1 min, there was a final 5 min extension period at 72 ℃ prior to a 4 ℃ hold. The amplified products were analysed by using electrophoresis on 1% agarose gel and photographed under UV trans-illumination after staining with ethidium bromide (0.5 μg/mL).

The amplification products were sequenced with the same primers. The sequences were edited and manipulated with the Molecular Evolutionary Genetics Analysis V6 (MEGA6) software. The sequences obtained were compared with those from GenBank using the BLAST program (Altschul, 1997).

Scanning electron microscopy morphology analysis

Scanning electron microscopy (SEM) studies were carried out to observe the cell morphology and structure of unknown isolated microalgae. Firstly, Cultured monospecies with 300 μL were filtered by applying light pressure on the plunger of a 10 mL syringe connected to small opaque plastic container, mounted on a filter nucleopore 25 mm a diameter (with pores of 2.5 μm). The filtered samples were dehydrated through passage over a series of alcohols in increasing concentration (50%, 60%, 70%, 80%, 90%, 100%)

and later isoamyl acetate 100% added in 10 min per ca. After the process of critical point drying, they were coated onto an ion coater for 220 s and observed under the SEM microscope (Hasle, 1978; Chen, 2007).

Growth rate measurement

Triplicate cultures of each monospecies were grown in tissue culture wells, containing 5 mL of sterile f/2 culture medium with no change culture condition, except at each of the five temperatures 18, 22, 26, 30 ^{o}C. Cell counts were made every 24 hrs (Neubauer haemocytometer), and all experimental cultures were harvested after 1 week. Specific growth rates were calculated as: (day^{-}1) = ln(F1/F0)/(t1–t0), where F=biomass at time of harvest t1 and F0=biomass at times zero, t0 (Guillard, 1973).

From the specific growth rate (μ), the number of cell divisions (n) per day was estimated using the following equation: n = μ/[ln(2)], where n is the number of cell divisions per day (Giovagnetti et al., 2012).

Results

Classification and isolation of microalgae

In the survey of the microalgae from the digestive tract of hard clam, total 34 species were determined, including 1 species of cyanobacteria, 8 species of chlorophyceae (8 genera), 2 species of dinoflagellate (2 genera), 21 species of diatom (12 genera) and 2 unknown species which were named U4 and U5. The microalgal species are listed in Table 1.

Among these microalgae, 2 unknown species were successfully isolated to monospecies (Figure 1)

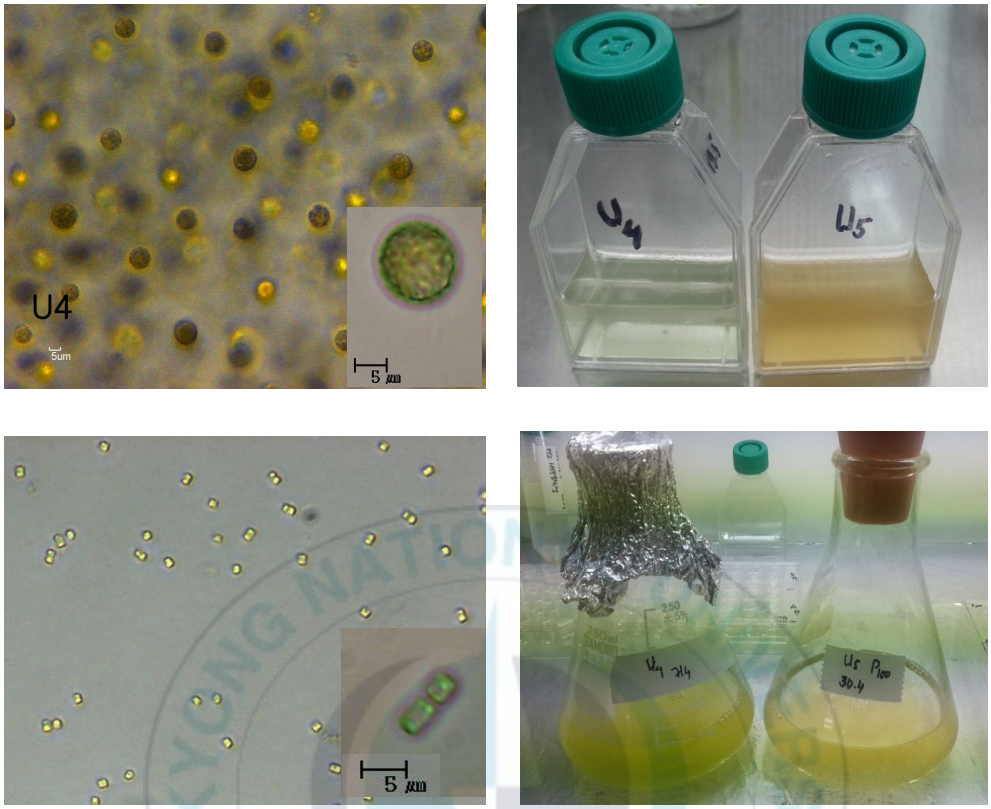

Figure 1. Isolated microalgae U4 and U5.

Table 1. The list of microalgae isolated from the digestive tract of hard clam

Diatom	***Cyanobacteria***
Actinoptychus undulatus	*Merimopedia sp.*
Cyclotella striata	
Cocconeis sp.	***Chlorophyceae***
Coscinodiscus excentricus	*Botryococcus sp.*
Coscinodiscus oculus-iridis	*Chlorella sp.*
Coscinodiscus radiatus	*Coelastrum* sp.
Coscinodiscus sp.	*Crucigenia tetrapedia*
Coscinodiscus subtilis	*Pandorina* sp.
Coscinodiscus wailesii	*Pediastrum duplex*
Cylindrotheca closterium	*Scenedesmus* sp.
Diploneis crabro	*Tetrastrum* sp.
Navicula rhynchocephala	
Navicula sp1.	***Dinophyceae***
Navicula sp2.	*Diplosalis sp.*
Navicula sp3.	*Prorocentrum micans*
Nitzschia sp1.	
Nitzschia sp2.	***Unknown***
Paralia sulcata	*U4*
Pleurosigma sp.	*U5*
Triceratium favus	
Trachyneis aspera	

Molecular identification of unknown microalgae

The result of the amplification of DNA polymerase gene of isolated microalgae were analysed by using electrophoresis on 1% agarose gel (Figure 2).

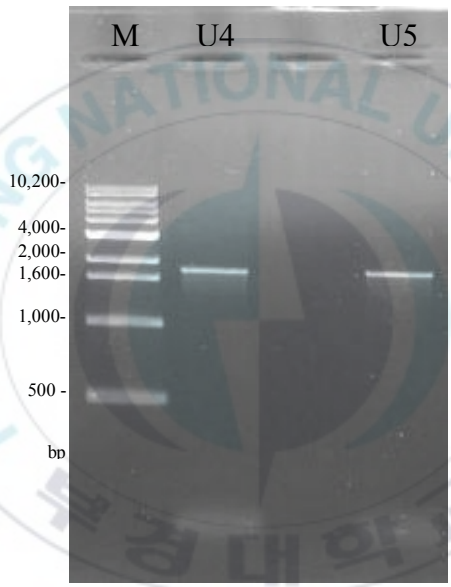

Figure 2. PCR amplification of the 18S rRNA gene of U4 and U5.

There were 807 and 806 nucleotides in the 18S rDNA gene sequences obtained from U4 and U5 samples respectively. After submitting to the National Center for Biotechnology Information (NCBI), the amplified 18S rDNA gene sequence of U4 was found to have 97% identity typical of that of previously sequenced *Neorhodella cyanea* (Figure 3) and named *Neorhodella* sp. In addtion, the result of sequence of U5 was found to be close to *Minutocellus polymorphus* strain CCMP497 with 98% similarity (Figure 4) and named *Minutocellus* sp.

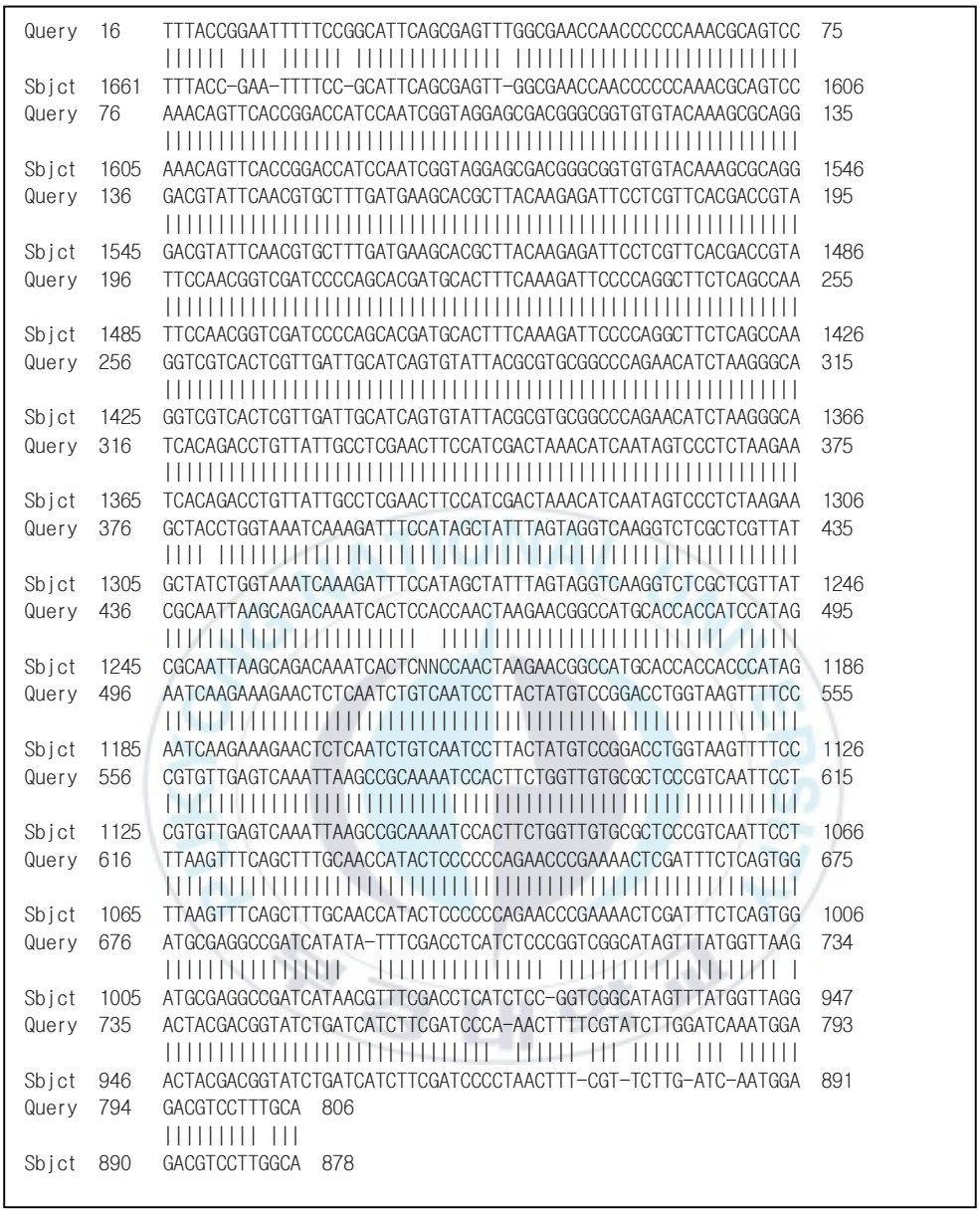

Figure 3. Comparison of 18S rRNA gene sequences of U4 sample and *Neorhodella cyanea* (AB649035.1).

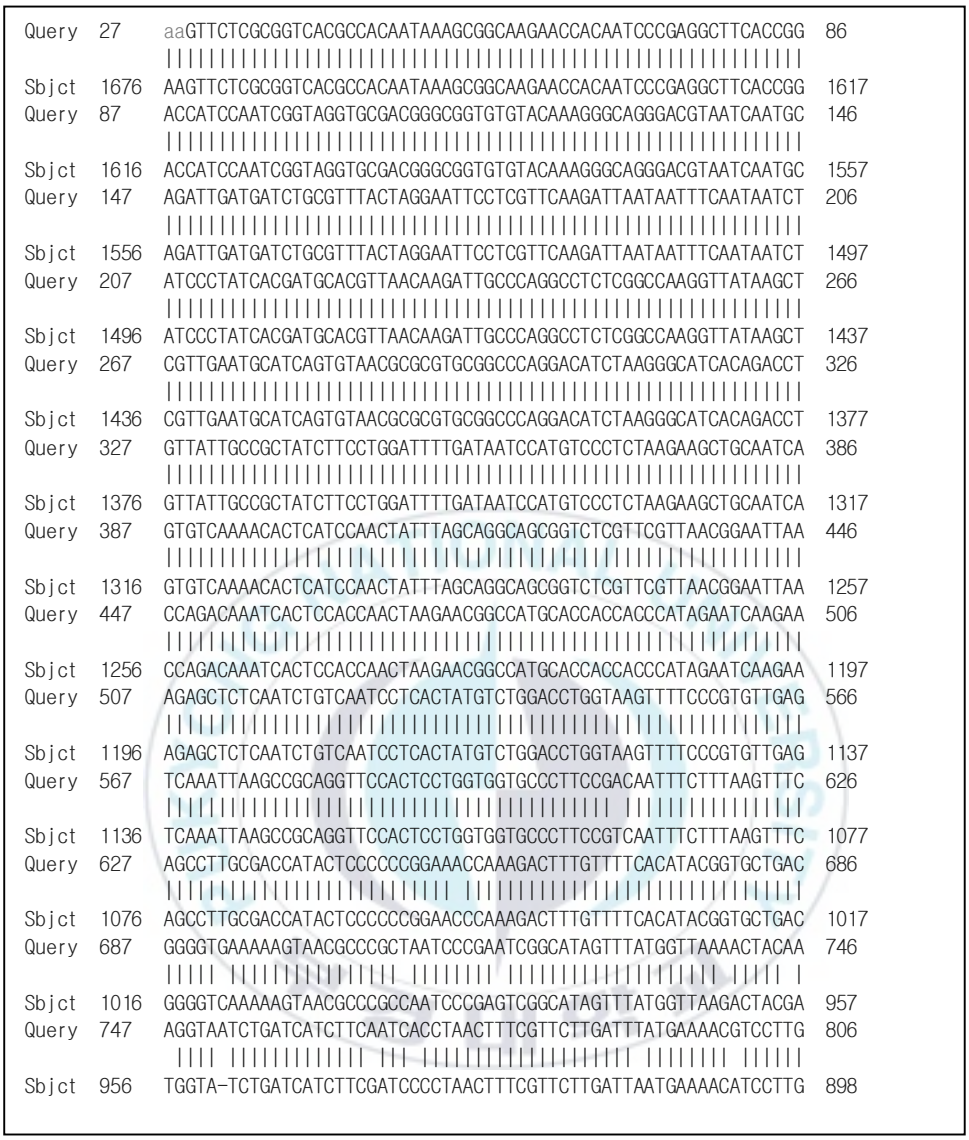

Figure 4. Comparison of 18S rRNA gene sequences of U5 sample and *Minutocellus polymorphus* strain CCMP497 (AY485478.1).

Scanning Electron Microscopy Morphology Analysis

Neorhodella sp. was solitary and spherical cells between 6 and 20 µm in diameter. They had a rough surface with mucilage (Figure 5).

Minutocellus sp. was solitary or united in chains. Cells were cylindrical with an elliptical to subcircular valve of (0.5x2.9x2 µm), with one convex valve and another concave. Knots on surface of valve convex made short chains (Figure 6).

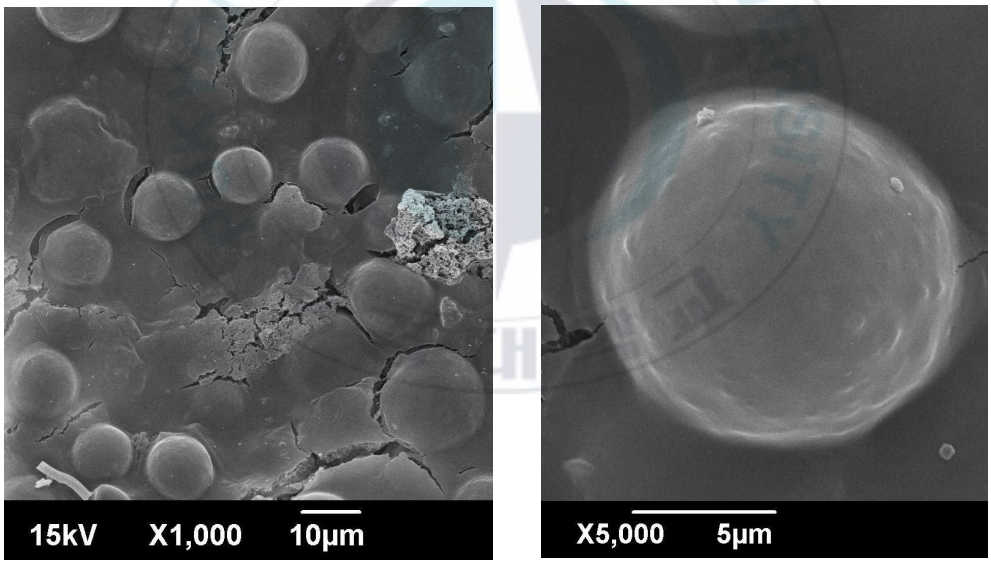

Figure 5. SEM picture of the isolated U4, *Neorhodella* sp.

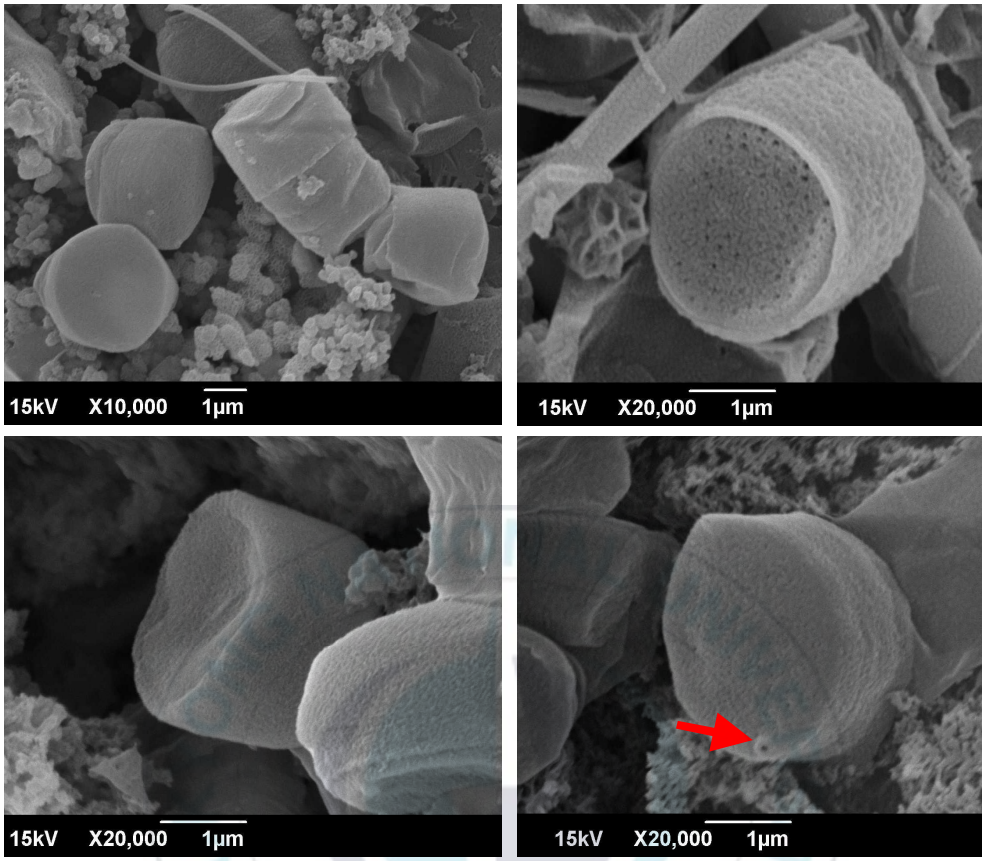

Figure 6. SEM picture of the isolated U5, *Minutocellus* sp.

Growth rate measurement

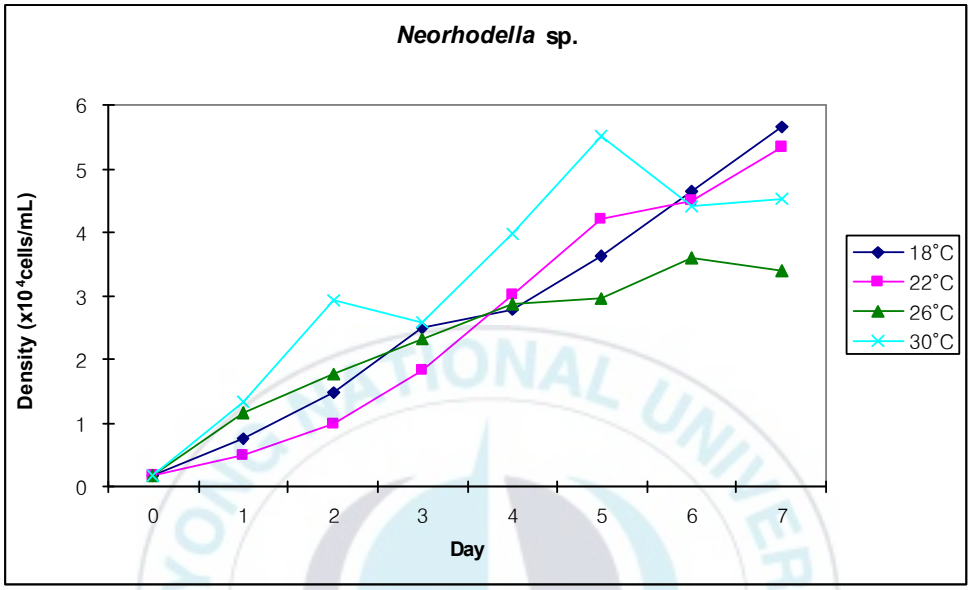

Figure 7. Growths of the isolated U4, *Neorhodella* sp. at three different temperatures

Figure 7 shows the results of *Neorhodella* sp. growth during a week at three different temperatures. An increase in culture temperature from 18 to 30 ^{o}C led to a increase in the density of Neorhodella sp. U4 from 0.17 x 10^4 cells/mL to 2.96±1.17 x 10^4 cells/mL at 26 ^{o}C and to the maximal mean cell concentration 5.52±0.23 x 10^4 cells/mL at 30 ^{o}C. However, after 5 days of

culturing, cell density fell sharply, at temperature 30 °C whereas that at 18 °C progressively raised with cell count of approximately 5,65±0.55 x 10^4 cells/mL and next 5,34±1.01 x 10^4 cells/mL at 22 °C.

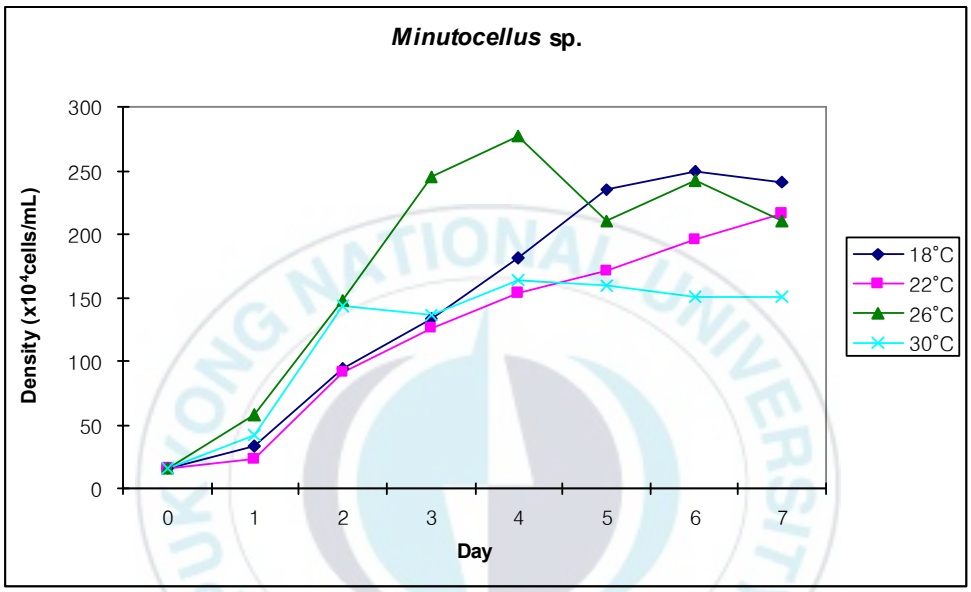

Figure 8. Growths of isolated U5, *Minutocellus* sp. at three different temperatures

In this study, the trend of of *Minutocellus* sp cell density. was on increase for about 4 days (Figure 8). And then, the cultured strain at 26°C declined rapidly from 276.5±0.54 x 10^4 cells/mL to 210.5±0.79 x 10^4

cells/mL. At the end of culture week, densities of *Minutocellus* at different temperatures remained steady, with the lowest cell count at 30 ^{o}C about 150.5±17.2 x 10^{4} cells/mL.

Table 2. Cell count at harvest and growth rate of Neorhodella sp. and *Minutocellus* sp. grown at three different temperatures (n=3)

Species	Temperature (°C)			
	18	22	26	30
Growth rate μ (day^{-}1)				
Neorhodella sp.	1.55±0.10	1.49±0.20	0.44±0.08	0.47±0.02
Minutocellus sp.	0.39±0.01	0.37±0.05	0.36±0.08	0.32±0.02
Cell divisions (day^{-}1)				
Neorhodella sp.	2.24	2.15	0.63	0.68
Minutocellus sp.	0.56	0.53	0.52	0.46

Neorhodella sp. and *Minutocellus* sp. grown at 18 ^{o}C obtained the highest specific growth rate 1.62±0.39 and 0.39±0.01 per day respectively (Table 2). Whereas, the lowest specific growth rate were *Neorhodella* sp. grown at 26 ^{o}C (0.45±0.13), and *Minutocellus* sp. grown at 30 ^{o}C (0.32±0.02). The number of divisions per day ranged between 0.63 and 2.24

divisions day^{-1} in *Neorhodella* sp. and between 0.46 and 0.56 divisions day^{-1} in *Minutocellus* sp. (Table 2).

Discussion

The results of 21 diatom species presented in digestive tract was showed that diatom was main food of hard clam *Meretrix lusoria*. This is consistent with the purpose of isolation and use diatom as feed for larvae and juvenile of Pacific oysters, Greenshell™ mussel, abalone (Ragg et al., 2010; Barille´ et al., 2003, Knuckey et al., 2002, Chen Y. C., 2007)

The Korea Marine Microalgae Culture Center (KMMCC) also used capillary pipette techniques and F/2 medium to isolate and grow the microalgal species sampled from Korean (Hur, 2008). However, they had three culture rooms at the temperature levels of 15, 20 and 25 °C while our samples were maintained in 18 °C. In addition, some species presented in this study were also cultured in KMMCC, such as *Cyclotella striata* (Kützing) Grunow, *Paralia sulcata* (Ehrenberg) Cleve, *Trachyneis aspera* (Ehrenberg) Cleve and *Prorocentrum micans* Ehrenberg.

Although morphological analysis is one of the important means, and very often used to identify common microorganism, it is not very accurate with special species which are small size and of simple morphology.

Therefore, like many other authors, this study applied molecular technologies by analyzing 18S rDNA to classify the species which could not be identified by their morphology (Soylu and Gonulol, 2012; Zhang et al., 2014; Liu et al., 2013). With above method, *Minutocellus* was identified in culture from the digestive tract of hard clam; consistent with previous findings of its presence in the upwelling region in South-East Pacific Ocean (Gall et al., 2008) and the Indian Ocean (Giovagnetti et al., 2012). This genus is belongs to diatom (Bacillariophyceae) and it's of very small size, picoplankton (about 3μm). Besides, the other unknown monospecies was identified as *Neorhodella* and had previously been isolated and cultured from marine and estuarine environments according to Scott et al., (2011). This genus belongs to red algae (Rhodellophyceae). More so, both genera identified by the study (*Neorhodella* and *Minutocellus*) were not listed strains cultured by KMMCC.

Billard & Fresnel, (1986), previously described details of *Rhodella cyanea* which was unicellular red algae with nuclei, chloroplasts and the conspicuous mucilage sheath. However, based on a small assortment of well-defined ultrastructural characters by TEM and coupled with recent molecular and LMWC analyses, Scott et al. 2008 believes that the cellular

features of *Rhodella cyanea* are distinguishing; to establish the new genus *Neorhodella*. *Neorhodella* sp. has smaller cell size than *Neorhodella cyanea*, ranging from 20 to 40 μm (Scott et al. 2008).

Minutocellus polymorphus that view lanceolate, elliptical to subcircular mantle low, apices somtimes rostrate with the pili had short branches near the base on valve convex (Round et al. 1990). Although the cultured species *Minutocellus* sp. had distinct characteristics described above, according to Round et al., (…..) this genus becomes almost circular as the cells decrease in size in culture. The number of copulae increase and some of the valve detail disappear. Harverages et al., (1989) reported that diatom *Minutocellus polymorphus* was one of five picoplanktons from Nagarransett Bay, and exudates from *Minutocellus* (clone MNL 100), negatively effected growth of other two diatoms (*Skeletonema* and *Asterionella*); log phase exudates inhibited the diatom *Asterionella* and the flagellate *Heterosigma*.

Two of the species *Neorhodella* sp. and *Minutocellus* sp. reached maximum growth rates at temperatures 18 ^{o}C, with slower growth and decline in cell numbers at temperatures above 26 ^{o}C. It has thus, been suggested that the optimum temperature range for best growth of two strains is from 18 ^{o}C to 22 ^{o}C. Acording to Giovagnetti et al., 2012, *Minutocellus*

RCC967 grew optimally under light conditions of 100 mmol photons m^{-2} s^{-1} at 20 ^{o}C, where it attained its highest growth rate (0.82±0.02) but later growth rate decreased under higher light. In contrast, the growth rate of *Minutocellus* RCC703 gradually increased over the light range, reaching its highest growth rate (1.97±0.15) at 500 mmol photons m^{-2} s^{-1}. Based on the result of growth rate, *Minutocellus* cultured 90 m^{-2} s^{-1} at 18 ^{o}C resembled *Minutocellus* RCC967. Study results indicated that the trend of the number of divisions per day of *Minutocellus* decreased when the temperature increased. However, all two strains *Minutocellus* RCC967 and RCC703 tend to increase the number of divisions per day when the light condition increases.

Conclusion

Thirty four microalgae species were isolated from the digestive tract of *Meretrix lusoria*. Among them, 32 species were identified by morphology observed under a light microscope. Two axenic monospecies were cultured successfully at temperatures 18 - 30ºC, with optimum growth rates attained at temperatures 18 – 22ºC. Therefore, it has been suggested that the best temperature range for the growth of the two strains is from 18 to 22°C.

These two monospecies were later identified as *Neorhodella* sp. and *Minutocellus* sp. based on the sequence of their 18S rRNA gene and confirmed by SEM results.

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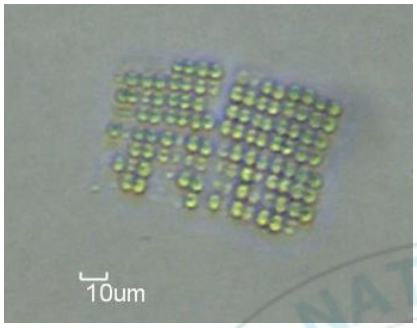
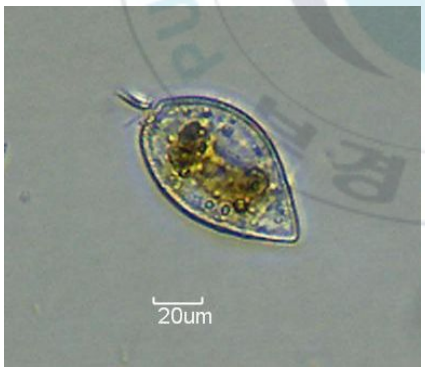
Appendix

Blue-green microalgae* (*Cyanobacteria)

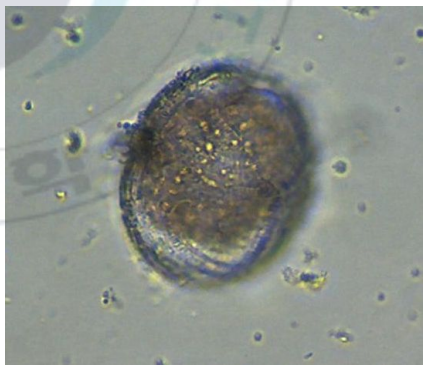


Merismopedia sp.

Dinoflagellate (Dinophyceae)

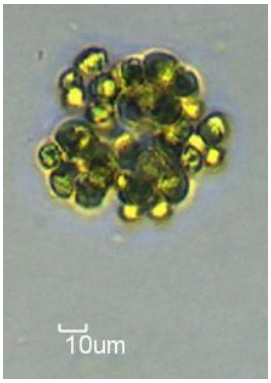


Prorocentrum micans Ehrenberg 1834

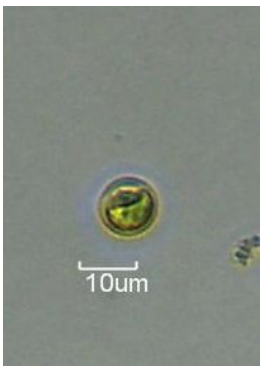


Diplosalis sp.

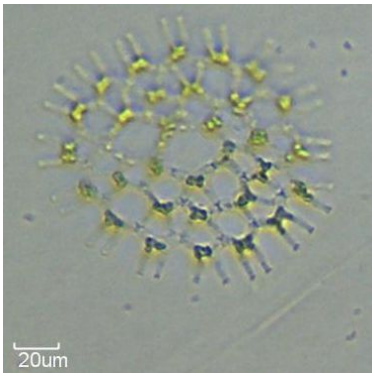
Green microalgae* (*Chlorophyceae)



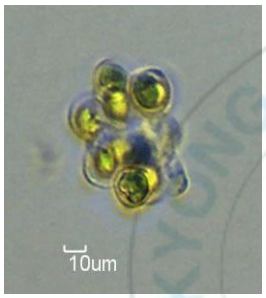
Botryococcus sp.



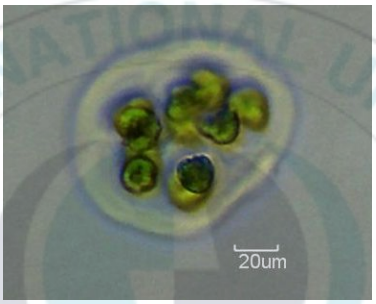
Chlorella sp.



Pediastrum duplex Meyen 1829

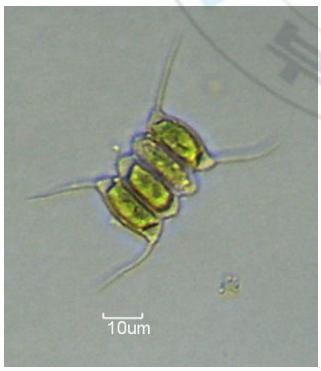


Coelastrum sp.



Pandorina sp.

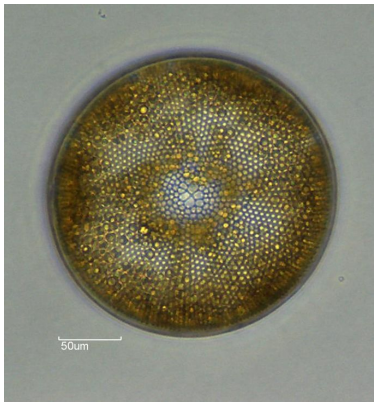
Tetrastrum sp.



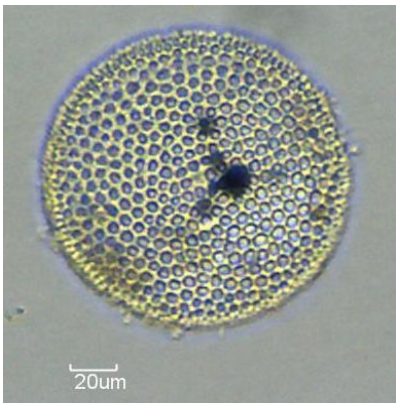
Scenedesmus sp.

Crucigenia tetrapedia (Kirchner) Kuntze 1898

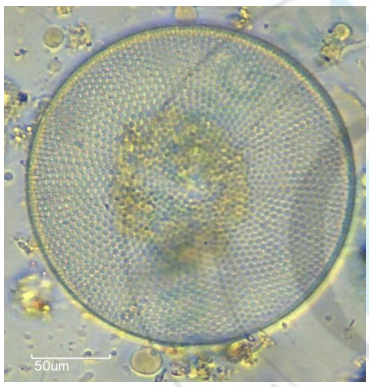
Diatom (Bacillariophyceae)



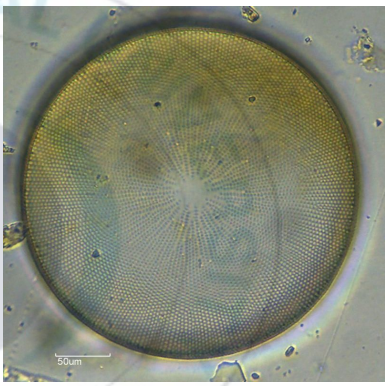
Coscinodiscus oculus-iridis Ehrenberg 1840



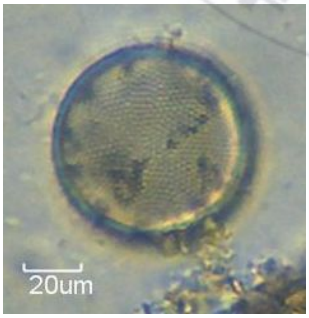
Coscinodiscus radiatus Ehrenberg 1840



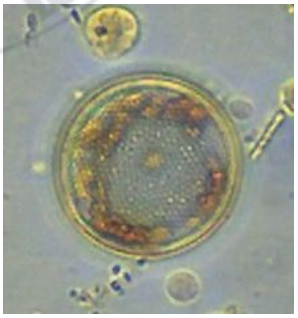
Coscinodiscus sp.



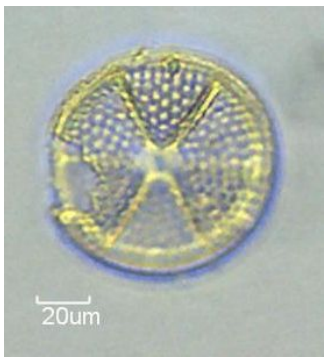
Coscinodiscus wailesii Gran & Angst 1931



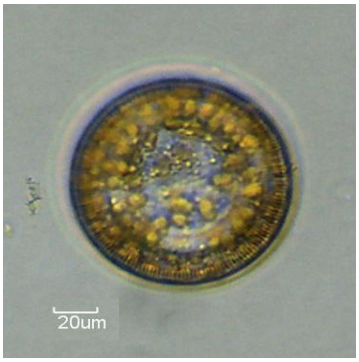
Coscinodiscus subtilis Ehrenberg 1841



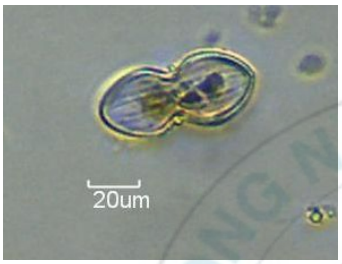
Coscinodiscus excentricus Ehrenberg 1840



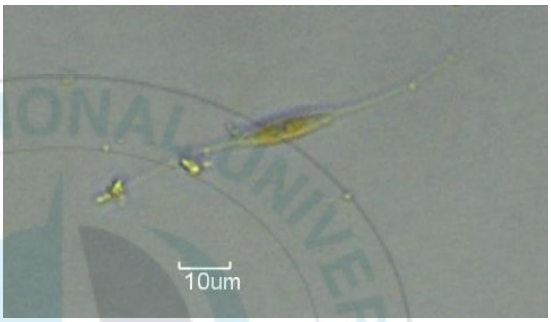
Actinoptychus undulatus (J.W.Bailey) Ralfs 1861



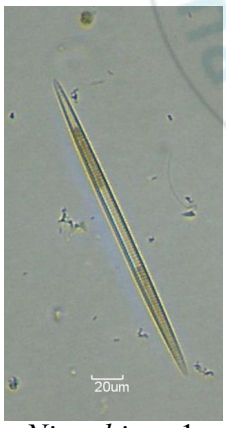
Cyclotella striata (Kützing) Grunow 1880



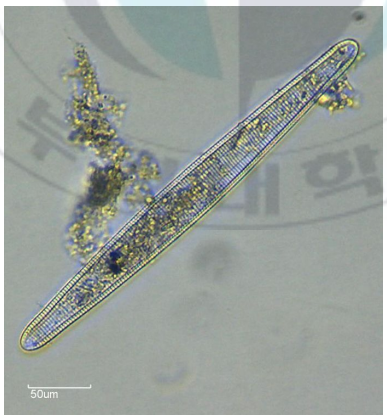
Diploneis crabro Ehrenberg 1854



Cylindrotheca closterium (Ehrenberg) Reimann & J.C.Lewin 1964



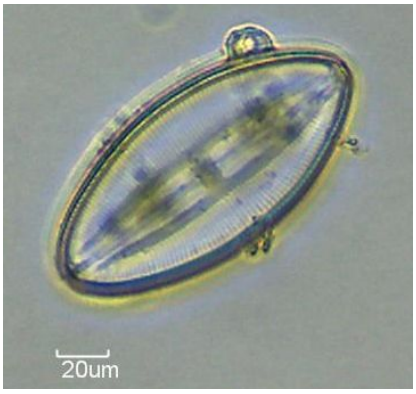
Nitzschia sp1.



Nitzschia sp2.

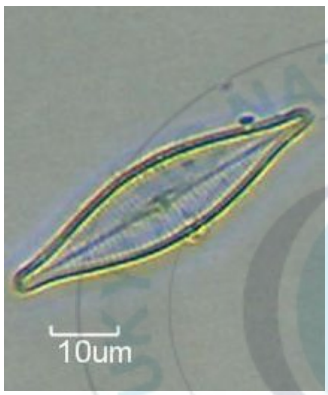
Pleurosigma sp.

Navicula sp1.

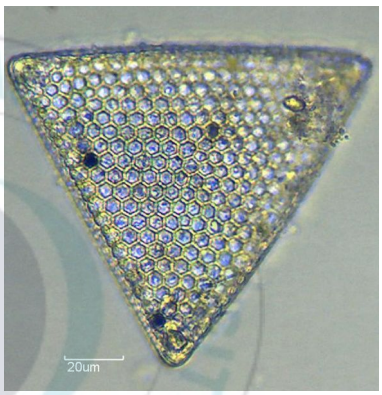


Navicula sp2.

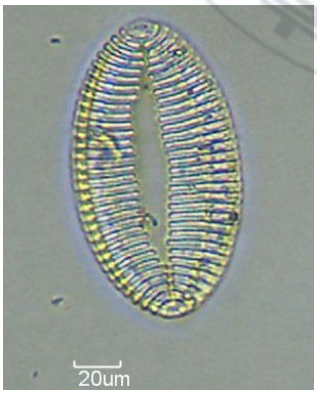
Navicula sp3.



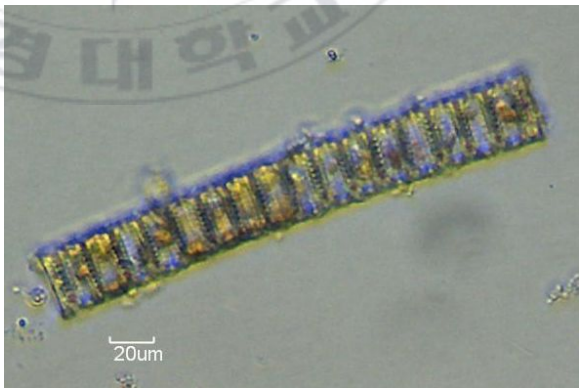
Navicula rhynchocephala Kützing 1844



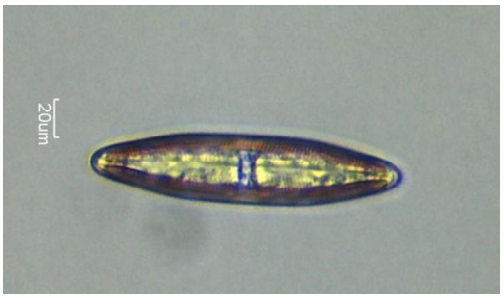
Triceratium favus Ehrenberg 1839



Cocconeis sp.



Paralia sulcata (Ehrenberg) Cleve 1873



Trachyneis aspera (Ehrenberg) Cleve 1894

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