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Hypothesis on Recurrent Bloom Formation by Cysts Germination of *Cochlodinium polykrikoides* in Southern Coastal Waters of Korea

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한국 남해안의 코클로디니움 포자발아에 의한 적조발생 가설

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Abstract

The study of resting and hyaline cysts of *Cochlodinium polykrikoides* has large ambiguities, though it has large importance on life cycle and ecology. In this study, It was observed the resting and hyaline cysts of *C. polykrikoides* in Jaran and Engang Bay during June 2010 and August 2012, respectively. The resting cyst was 35-43 μm in size, spherical to round and armored with developed colorless cell wall, possibly comprised of three layers. A conspicuous red body was typically observed. The early germinants were 35-45 μm long and 20-30 μm wide, and their vegetative cells developed into 8 chains at the maximum. The hyaline cyst was surrounded by a transparent, thin hyaline membrane and similar to vegetative cells in size, pale in color and immobile inconspicuous chloroplast. It is hypothesized that the existence of resting and hyaline cysts of *C. polykrikoides* and their successful germination processes can act as a local seed-bed responsible for recurrent blooms in Korean coastal waters.

Key words : *Cochlodinium polykrikoides*, Resting cyst, Hyaline cyst, Recurrent bloom

Ⅰ. Introduction

Certain dinoflagellates(~200 species; Head, 1996) have the ability to enter into a dormant or resting stage during sexual reproduction processes(Pfiester and Anderson, 1987), as the only diploid phase in their relatively complicated life cycle. These dormant stages called resting cysts that have peculiar characteristics: they are non-motile and are surrounded by a dense, resistant wall(Matsuoka and Fukuyo, 2000). The resting cysts have an ecological role as the source seedlings of the recurrent blooming and expansion of geographical distribution because it can survive in harsh environmental conditions. While some key dinoflagellate resting cysts causing harmful algal bloom(HAB) had been studied in many areas of the world(Matsuoka and Fukuyo, 2003), whether *Cochlodinium polykrikoides* Margalef produces resting cysts has been an open question(Fukuyo, 1982; Matsuoka, 1987; Kim et al., 2007; Richlen et al., 2010).

The mixotrophic dinoflagellate *C. polykrikoides* is a causative agent of the recurrent HABs observed in the southern and eastern coastal waters of South

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Korea. Massive *C. polykrikoides* related fish kills have been recorded with losses of US $ 60 million in 1995 and US $ 5-20 million per year in 2000-2007(NFRDI, 2007). Prior extensive studies (Kim et al., 2002; Kim et al., 2007; Lee and Lee, 2006; Park et al., 2005; Park et al., 2013), including NFRDI's comprehensive surveys, had been conducted on physical, chemical and biological parameters in HAB infected southern inshore waters of the Korean peninsula between 1997-2014, but the identity of the resting cyst was not fully confirmed in those studies. For example, Kim et al.(2007) identified multiple types of *C. polykrikoides* in the laboratory, including the well described athecate vegetative cells, armored vegetative cells (before and after bloom), resting cells and cysts. Park and Park(2010) detected *C. polykrikoides* in surface sediments of the southern coasts of Korea, using species-specific real-time polymerase chain reactions (PCR) technique and suggested that this species may persist in form of cyst in sediments where its blooms occur annually. It is remarkable that Tang and Gobler(2012) recently provided the clear visual evidence of the production of resting cyst, including sexual mating cell pairs, planozygotes with two longitudinal flagella, and time series micrographs of the cyst formation, and cyst germination processes from US estuaries. However, it is hypothesized that there will have large morphological differences between US and Korean *C. polykrikoides's* resting cyst based on Iwataki et al.(2008) classification. Actually, Iwataki et al.(2008) identified three distinct sub-clades or ribotypes, in *C. polykrikoides*; the East Asian ribotype (also referred to as Japanese-Korean), the American/Malaysian ribotype, and the Philippines ribotype. Afterall, the first morphological features of resting cysts of *C. polykrikoides* collected from Korean coastal sediments and evidence for the existence of resting cysts based on the morphological and molecular phylogenetic data were clearly provided by Li et al. (2015). The morphology of the resting cysts differed from that reported previously in sediments and culture experiments.

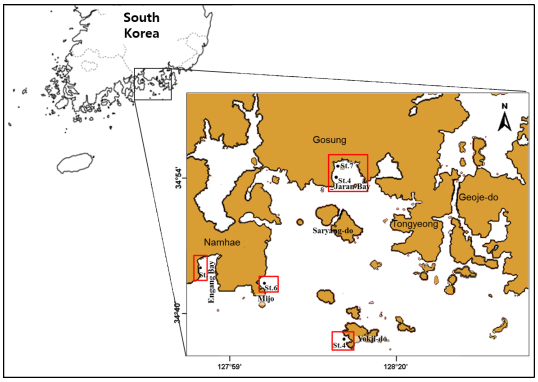
In additions, the recurrent *C. polykrikoides* blooms in Korea might be due to the formation of hyaline cyst(Kim et al., 2002), as a kind of temporary cyst formed by modification of vegetative cells without sexual reproduction. The hyaline cyst successfully turned into motile cells when it moved into the light and higher temperature(Kim et al., 2002; Kim et al., 2007; Shin et al., 2017). Shin et al.(2017) reported the formation of temporary cysts from the freshly collected field samples by using sediment trap for short period and observed sucessful germination in daily intervals, but there is a quessition that these temporary cysts can play a role for recurrent blooming seed after dormancy in the cold bottom coditions. Actually, the hyaline cysts proceeded from temporary cysts of *C. polykrikoides* were observed to survive up to 6 months at 4℃ in the dark in a laboratory culture(Kim et al., 2002).

However, the field observation of hyaline cyst is quite challenging perhaps due to rapid transformation from motile cells to hyaline cyst or vice versa. Therefore, the detail observations of resting and hyaline cysts of *C. polykrikoides* are needed. The purpose of the study is to investigate the resting and hyaline cysts of *C. polykrikoides* in surface and sediment and how they are related to the recurrent blooms in Korean coastal waters. The hypothetical blooming mechanism of *C. polykrikoides* related to resting and hyaline cyst is also discussed.

Ⅱ. Materials and Methods

Seawater samples(1 L) were collected during June to August 2010 in Jaran Bay; it is characterized by lower tidal current velocity(~0.1 ms-1; NORI, 2008), and its south part is opened to offshore and other parts are surrounded to land such as Sacheon and Goseong([Fig. 1]). The collected seawater was fixed with Lugol's solution and used to identify and count species with an inverted microscope at ×200-400 magnification. Net samples were also collected without fixation for resting cyst observation and isolation.

To investigate of hyaline cysts, samples were collected from Engang Bay during August 2012 before and after typhoon Bolaven(August 27-29, 2012).



[Fig. 1] Sampling sites of *Cochlodinium polykrikoides* cysts along southeastern coastal coast of Korea.

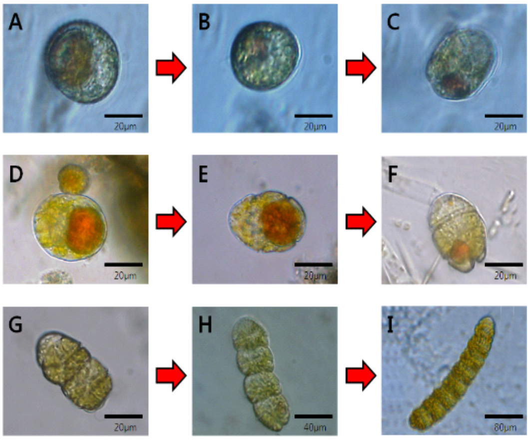
Samples were collected in screw-capped opaque bottles, transported to the laboratory and stored at 20℃ in a culture room with the caps left opened. The cells were monoclonally isolated and 20-30 cells were transferred to a 48-wall plate(Nunclon) containing f/2-Si medium(Guillard and Ryther, 1962) and sterile sea sediment and seawater collected at Jaran Bay. The plates were incubated at 20℃ on 14:10 h light-dark photoperiod at 100 μmol photons $m^{-2}s^{-1}$ with cool white bulbs and examined daily under microscope. Bottom sediments were collected using a core sampler(core pipe, diameter 10 cm). The upper 1-cm of the sediments was subjected to sonication for 30s and size-fractioned using 15-120 μm pore-sized sieves. The palynological processing method of Matsuoka and Fukuyo(2000) was used for initial treatment of the sediments.

Ⅲ. Results and Discussion

1. Resting cyst :

The resting cysts of *C. polykrikoides* were first found in the net sampling at the periodic examination on the St. 4 in Jaran Bay from the end of May through early June, 2010 and their germination process was verified under the microscope. Their size was 35~43 μm long and shape was spherical to rounded([Fig. 2 A, B]).

This morphology of resting cysts is quite different from that of Kim et al.(2007) that was rounded but folded at one side and flat, although they did not confirm the existence of resting cysts. In this study, resting cysts were observed to be with a small spine just before germination and had a conspicuous red body, which is nearly similar with the resting cyst verified from Korean coastal sediments(Li et al., 2015). These surface projections are different from previous reports(Fukuyo, 1982; Matsuoka and Fukuyo, 2003; Kim et al., 2007; Rubino et al., 2010). Fukuyo(1982) and Matsuoka and Fukuyo(2003) described cysts with prominent surface projections up to maximum to 7 μm and and an irregular shape(subspherical, ovoidal or ellipsoidal) that might be caused by biotic or chemical processes on the surface in the sediments or by the sampling processing.



[Fig. 2] Field observation of the morphology and germination processes of resting cyst of *C. polykrikoides* in Jaran Bay, Korea. (A-C) the morphology of resting cyst of *C. polykrikoides*, (D) resting cyst just before the germination process, (E-F) the resting cyst germination process (i.e. the transformation process of planomeiocyte), (G-I) the formation process of vegetative cells, [2-chain (G), 4-chain(H) and 8-chain (I) vegetative cells].

The morphology of resting cyst is quite different than hyaline cyst because it has colorless cell wall, possibly composed of three layers, while the hyaline cyst were surrounded by a transparent, thin hyaline membrane. Matsuoka and Fukuyo(2000) mostly founded resting cyst with a single cell wall and sometimes two layers. A conspicuous red body was found in the resting cyst. In contrast, Tang and Gobler(2012) reported resting cysts containing one or sometimes two red accumulation bodies and Rubio et al.(2010) founded living resting cyst with granular content and a central red body in the Mediterranean.

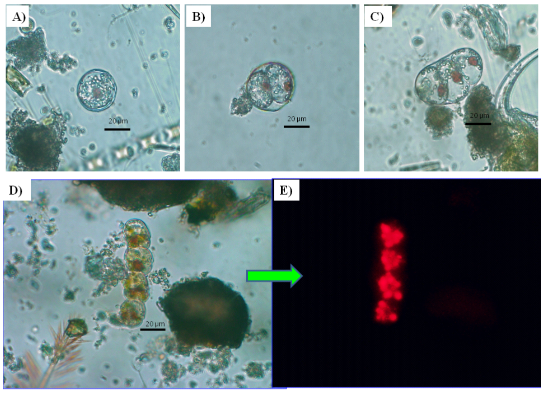
In this stage, the cysts started germinant asexually. Just before germination, the pigmentation of resting cysts became heavier with a large conspicuous red body. Initially, two sulcus and one cingulum were starting to develop, although small size red body was still present. A division furrow already observed in this stage with two tiny flagella as because it seemed as swimming stage. Two-cell germlings were observed. The early germinants were 35-45 μm long and 20-30 μm wide. Previously, Kim et al.(2007) also observed the development processes from an armored type into planktonic unarmored vegetative cells at laboratory. Moreover, Tang and Gobler(2012) observed detailed time series of the germination processes of *C. polykrikoides* resting cyst at 18℃ at laboratory. They stated that the germination of resting cysts occurred within the cyst wall in 8-31 days and followed some steps such as the surface became coarse after day 5, the cingulum became discernable on day 8, a division furrow was developed on day 16, two divided but connected germlings were developed on day 19 and the two new germlings were left behind an empty cyst during days 26-28. On the other hand, this study provided field sample observation. Kim et al. (2007) also mentioned that the armored type of *C. polykrikoides* has sufficient time to form blooms for ~2 months before the first red-tide.

In Jaran Bay, high abundances of resting cyst and vegetative cells of *C. polykrikoides* had been observed during June and mid-July, respectively. It is assumed that favorable environmental conditions (i.e. temperature >25℃, salinity >30 PSU) triggered resting cyst to germination. Generally, temperature is the major factor regulating the germination of marine phytoflagellate cysts(Dale, 1983; Ishikawa and Taniguchi, 1996) that varied between regions to regions. For instance, the lower temperature (18℃ in comparison to 21℃) and addition of antibiotics significantly increased the germination rate in US coasts and most cysts germinated successfully at different rates at 15℃ and 25℃ in red sea, Saudi Arabia. Similarly, Kim et al.(2007) observed germination at culture room temperature (20-25℃), and Li at al.(2015) isolated the germinated cells from the resting cysts inoculated into well plates at 20℃. Moreover, increase in temperature may act to prevent seeding or the maintenance of blooms in the water column during summer periods(Genovesi et al., 2007).

2. Hyaline cyst :

The hyaline cysts of *C. polykrikoides* were first found in the net samples of Engang Bay, Namhae at August 26, 2012(before typhoon Bolaven during August 27-29, 2012). It was pale in color, immobile with inconspicuous chloroplasts([Fig. 3A-C]). All hyaline cysts were surrounded by a transparent, thin hyaline membrane. Hyaline cells developed diversely into single cells or cell clusters being chained with two, three or four cells. Each cell had a red body, and cell clusters formed a hyaline membrane surrounding cells. Hyaline cells could be detected by microscopic observation without staining. They were determined to be alive under the fluorescence microscope([Fig. 3D-E]). It also suggested that hyaline cells could be considered a kind of temporary cysts, not to be at the dying stage of vegetative cells.

Hyaline cysts of *C. polykrikoides* were previously reported by Kim et al.(2002). They recorded single- and three-celled chains of temporary cysts formed following incubation in the dark for 6 months. the germination of hyaline cysts of *C. polykrikoides* occurs within 24 h. They examined the germination of a single four-celled chain of temporary cysts isolated from laboratory culture, and reported that individual vegetative cells germinated one by one from the chain-forming temporary cysts. However, Kim et al.(2002) conducted this experiment using a single four-celled chain of temporary cyst after it was maintained for 6 months at 4℃ in darkness. This long term storage in darkness and at low temperature may have altered cell contents or the metabolic activity of temporary cysts. The single-celled temporary cysts were also reported, previously(Tomas and Smayda, 2008; Tang et al., 2012).



[Fig. 3] Field observation of the morphology of hyaline cysts of *C. polykrikoides* (A-D) in net samples of Engang Bay, Namhae during August 26, 2012. The hyaline cells were photographed by LM (D) and fluorescent microscopy (E) with a clear red fluorescent response on chloroplast.

However, the differences between resting and hyaline cysts were lies on mainly two factors: firstly, all hyaline cysts were surrounded by a transparent and thin hyaline membrane, and in contrast, resting cysts were surrounded by permanent thick cell wall(s), and, secondly, hyaline cysts transform to the vegetative stage in a short time and, for example, when the temperature of 10-15℃ was abruptly increased to 25℃, the isolated types developed into vegetative cells with a large red lipid body (red body) with 2 h(Kim et al., 2007). In contrast, the germination of a resting cyst takes place within the cyst over a longer time (about a month) with the germling leaving the cyst through a cryptopylic archaeopyle(Tang and Gobler, 2012). In laboratory, the formation of hyaline cysts was higher at 23℃ than 17℃, and 20℃ with 4 cell-chain(Shin et al., 2017). The abundance of hyaline cysts was higher in Gosung Bay, comprise up to 41% of the total cyst assemblage, compare to Buk Bay and other sites where they present in low proportions(Pospelova and Kim, 2010). Recently, Shin et al.(2017) examined the factors affecting the formation and germination of temporary cysts of *C. polykrikoides*, and provides details about the germination process. In the laboratory experiments, *C. polykrikoides* produced the chain-forming temporary cysts that are immobile and surrounded by a hyaline membrane as shown in the result of Kim et al.(2002). The encystment indicated that darkness induces the formation of chain-forming temporary cysts, consistent with field observation by using the sediment trap experiment. Germination occurred twice from a single four-celled temporary cysts within 24 h after exposure to light, and the germlings appeared as two-celled chain-forming vegetative cells. The germination behavior of temporary cysts of *C. polykrikoides* differs from that of other dinoflagellates, and this may be a survival strategy for the maintenance of population size during dense blooms.

3. Vegetative cells :

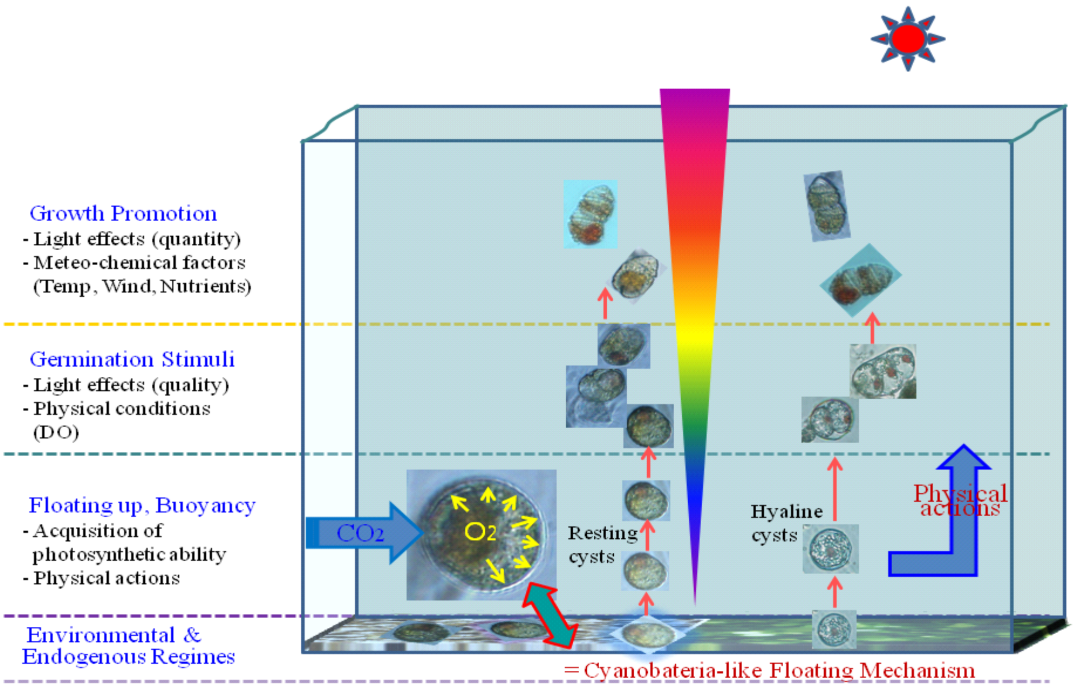
In this study, the size of vegetative cells (35-43 µm in cell length) is somewhat similar with Japanese-Korean ribotypes of *C. polykrikoides* (30-40 µm in cell length) (Iwataki et al., 2008), smaller than the type specimen of Puerto Rico (approximately 50 µm in length) called as Malaysian-American types (Matsuoka et al., 2008). However, this vegetative cell is larger than Kasasa (27-30 µm in cell length), newly identified in Japan that is also characterized by the following features: cells are ellipsoidal in outline with a conical epicone and a hypocone with two well-developed antapical lobes due to the deeply indented sulcus end. However, all ribotypes of *C. polykrikoides* are smaller than *C. fulvescens* (Iwataki et al., 2007) that has larger size, ranging 37.5-57.5 µm(mean 45.8 µm) in length, 30-42.5 µm (mean 35.5 µm) in width, and has yellowish brown granular chloroplasts, the sulcus running between the cingulum on the dorsal surface. In this study, the vegetative cells can form 8 chains; that is larger than Kasasa, the number of cells in chains in a maximum of two.

4. Blooming concept :

The exact dormancy period for *C. polykrikoides* resting cyst in Korean coasts is still unknown. Generally, the duration of this “maturation” process is highly variable among species ranging from 12 h to 12 months(e.g. Pfiester, 1977; Pfiester and Anderson, 1987, Perez et al., 1998) and it can differ with a single species when storage conditions changes(Anderson, 1980; Montreson and Marino, 1996). It is assumed that bloom-enhancing temperature exaggerate resting cyst and this process is firstly marked by appearance of red fluorescence due to chlorophyll that is synthesized in germinating cyst (Anderson and Keafer, 1985) and subsequently by the appearance of distinctive germling cells (planomeiocytes) in the water column (Anderson et al., 1983) with the presence of optimum oxygen level. Thus, it is hypothesized that by the presence of optimum light quality, temperature and oxygen, associated with lifting agents (i.e. tidal current, winds, Fig. 4), the germling cells of *C. polykrikoides* can be floated in the upper surface sediment. This process is particularly similar to cyanobacterial buoyancy regulation processes. This is evidence that by regulating cyanobacterial buoyancy according to light intensity, it can perform a daily vertical migration (Walsby and Klemer, 1974; Konopka et al., 1978). However, the successful germination cells of *C. polykrikoides* could create blooms with appropriate bloom-enhancing environment (water temperature of 20-28.4℃ and salinities of 16.34-30.63 PSU; Lee and Kim, 2008) in Korean coastal waters. The existence of hyaline cysts in Engang Bay, Namhae put forward another blooming mechanism([Fig. 4]).

This concept might be involved that bloom forming temperature and light stimulation are enhancing hyaline cysts germination. Kim et al.(2002) reported that, after being preserved for six-month at 4℃ in darkness, *C. polykrikoides* motile cells regenerated successfully when transferred to 20℃, a photon flux density of 40 μmol photons $m^{-2}s^{-1}$, and 14:10 h light-dark photoperiod. The formation of hyaline cysts in the life cycle of *C. polykrikoides* may act as temporary cysts or an overwintering survival strategy.

However, It is hypothesized about the possible blooming mechanism in Engang Bay that sudden



[Fig. 4] Hypothetical diagram of blooming mechanism of *C. polykrikoides* in Korean coastal waters.

increased temperature were enough to hyaline cyst germination and bloom formation, and consequently, blooms disappeared after typhoon Bolaven during August 27-29, 2012. Lee(2008) stated that *C. polykrikoides* blooms were obviously terminated in response to either the strong northeasterly storms(1995, 1996 and 1999) or the typhoon incursions(1997, 1998 and 2003).

5. Conclusions :

From the above discussion, the field observation of resting and hyaline cysts of *C. polykrikoides*, and their successful germination processes can act as a local seed-bed responsible for recurrent blooms in Korean coastal waters. Future research are needed to concern about some topics such as (1) field observation of planozygote, (2) determination of exact dormancy period, (3) understanding complete life cycle, and (4) extensive surveys for yearly cyst maps of *C. polykrikoides*.

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