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Reproductive behavior and role in maintaining an aggregative form of the freshwater green alga Marimo, *Aegagropila linnaei*, in Lake Akan, Hokkaido, Japan

- **Author names and affiliations.**

Taketo Umekawa[a]*, Isamu Wakana[b] and Masashi Ohara[a]

[a] Graduate School of Environmental Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan

[b] Kushiro International Wetland Center, Kushiro, Hokkaido 085-8505, Japan

* Corresponding author. Tel: +81 90 7659 1190

*E-mail address:* umekawataketo@gmail.com

## Abstract

*Aegagropila linnaei* is a unique freshwater green alga that develops into spherical aggregations known as “lake balls”, “*Cladophora* balls” or “Marimo”. Loss of this species is progressing globally, and there is concern over threats and its conservation. As part of the study which aims to conserve this endangered species, zoosporogenesis of *A. linnaei* in Lake Akan, designated as a Special Natural Monument of Japan, was investigated. Materials encompassing three growth forms of *A. linnaei*: "aggregative", "free-floating", and "epilithic", were collected at five sites around the lake at regular intervals from spring to autumn of 2017 and 2018. Quadriflagellate zoospores were observed at three sites (one aggregative and two epilithic) in mid-August and early September with a reproducibility, but not at two sites (one aggregative and one free-floating). Percentages of zoospore-producing filaments were extremely low (maximum 1.3%), and the result of statistical analysis by generalized linear model showed no significant difference among the study sites and periods in both years. These results indicated that the zoosporogenesis of *A. linnaei*, which has been thought to be an extremely rare event in the past, occurs regularly even though being low percentage. Such rare zoosporogenesis appears to provide a few initials for the ensuing generations and to contribute to maintaining the

aggregative form through the continuation of vegetative growth.

**Keywords:** Conservation, Growth form, Lake ball, Reproduction, Spherical aggregation, Zoospore

## 1. Introduction

*Aegagropila linnaei* Kütz. (Cladophorales, Ulvophyceae) is a perennial freshwater alga that is widely distributed over the northern hemisphere (Hanyuda et al., 2002; Leliaert and Boedeker, 2007; Boedeker et al., 2010a; b). An individual of this species is a 1–4 cm long filamentous thallus called a “filament”, and three growth forms have been recognized depending on morphological and ecological state of the filament(s) (John, 2002; Soejima et al., 2009; Boedeker et al., 2010a; b): “aggregative” being a mass of radially arranged filaments, “free-floating” as a filament thought to be free from the aggregation (Kurogi, 1980) and “epilithic” attached onto rock or other substrates with rhizoids. Spherical aggregations sometimes become as big as a human head, so they are called “lake balls” (van den Hoek, 1963), “*Cladophora* balls” based on a previous genus name (John, 2002), or Japanese name “Marimo” (Kurogi, 1980). Remarkable progress has been made in terms of understanding the phylogeny and taxonomy (Hanyuda et al., 2002; Boedeker et al., 2012), biogeography (Boedeker et al., 2010b), population ecology (Einarsson et al., 2004; Soejima et al., 2009), photosynthetic physiology (Cano-Ramirez et al., 2018), morphology and development (Horiguchi et al., 1998; Togashi et al., 2014) of this species. In contrast, *A. linnaei* is declining globally, and concern over its status and conservation is surging in many countries (Wakana, 1999; Boedeker et al., 2010a).

Lake Akan, located in eastern Hokkaido, Japan, is one of the best known habitats where large spherical aggregations grow gregariously (Kurogi, 1980; Wakana, 1999). In this lake, the aggregations have a dense and quite beautiful surface; their maximum diameter often exceeds 30 cm (Kurogi, 1980; Wakana and Nakayama, 2017). These characteristics were regarded as a symbol of nature in Japan, such that *A. linnaei* in Lake Akan was designated as a natural monument of Japan in 1921; then as a special natural monument in 1952 by promotion of status. However, in the first half of the 20th century, *A. linnaei* populations were remarkably reduced due to inflow and sedimentation of soil and sand caused by deforestation. In the latter half of the century, the number of surviving aggregations was down by half due to eutrophication brought about by polluted water that flowed in from dwellings and tourist facilities around the lakeside (Wakana, 1999). Therefore, various countermeasures including regulation of deforestation by law and cleanup of the lake water through servicing of public sewage have been conducted (Wakana, 1999). Unfortunately, despite these efforts, there is no evidence of recovery of *A. linnaei* populations. To further advance the conservation approaches, it is necessary to increase knowledge of this species, especially regarding its growth and reproduction.

As a major event of reproduction, there have been a few prior reports about formation of biflagellate zoospores, using cultured materials from Lake Akan (Nishimura and Kanno, 1927; Yabu, 1975). Many species of the order Cladophorales of which *A. linnae* is classified as a member generally produce by alternating isomorphic generations through biflagellate and quadriflagellate zoospores (Bold and Wynne, 1985). A gametophyte produces biflagellate zoospores, and male and female zoospores conjugate and grow into a sporophyte which resembles the gametophyte. This sporophyte produces asexual quadriflagellate zoospores which grow into gametophytes without conjugating. Therefore, the previous studies conducted under the culture may have revealed only a part of the reproductive behavior in *A. linnaei*. In this paper, we tried to verify natural zoosporogenesis of this species in Lake Akan.

## 2. Materials and methods

Lake Akan (center: 43°27′N, 144°06′E, boundary length: 30.3 km, surface area: 13.3 km$^2$ and mean depth: 17.8 m) is a freshwater lake of Akan Caldera. *A. linnaei* is distributed along the coast of this lake and 14 populations have been found (Wakana and Nakayama, 2017). In this study, we selected five major populations as the study sites: Churui and Kinetanpe for aggregation, Takiguchi for free-floating, and Jyagaiwa and Shurikomabetsu for epilithic forms (Table A1, Fig. A1 and A2 in supplementary material). Churui is a bay with sandy lakeshore and inflowing rivers. Kinetanpe is similar to Chuluai, but the lakeshore is marshy and the substrate is muddy. Takiguchi is a small and shallow bay with lava substrate, and flow environment is relatively moderate due to influence of the opposite island. Jyagaiwa is also a small bay, but substrate is gravel by wave action. Shurikomabetsu with a north-facing flat lakeshore is sandy gravel substrate and falls under shadow of trees of the lakefront in the daytime.

Six samples of each type of aggregation, rocks or small masses of free-floating filaments were collected at the five sites above by snorkeling or scuba diving at regular intervals from spring to autumn excluding the freezing season of 2017 and 2018. Fifty filaments per sample (in total 300 filaments per site) were isolated, and the number of zoospore-producing filaments was counted under a microscope (Nikon optiphot-2, camera: COOLPIX995). Statistical relationship between the percentage of zoosporogenesis and the study sites and period was tested by generalized linear model (GLM) using R (Dobson and Barnett, 2008). The number of zoospore flagella was checked after fixing in Lugol’s solution (Chihara and Hara, 1979).

## 3. Results and discussion

In this study, we confirmed the formation of zoospores in natural materials of *A. linnaei* from Churui, Jyagaiwa and Shurikomabetsu in mid-August and early September with a reproducibility (Fig. 1a, Table 1). In Shurikomabetsu, the zoospore formation continued until late October 2017. They were teardrop or straw bag shaped with an eye-spot and four flagella (Fig. 1b, c), and were 10–30 μm in diameter. As shown in Table 1, the percentages of zoospore-producing filaments were extremely low and the maximum was only 1.3%. In addition, no zoospores were observed in the materials from Kinetanpe and Takiguchi. Because of such low percentages and their narrow dispersion, result of statistical analysis by GLM showed no significant difference between the percentages of zoosporogenesis and the study sites as well as periods in each year of 2017 and 2018.

Zoosporogenesis in *A. linnaei* was reported by Nishimura and Kanno (1927), and Yabu (1975). They observed a series of processes of formation, discharge, adhesion, and germination of zoospores with two flagella in July–August of 1927 and September–October of 1974, respectively, using cultured aggregations collected from Lake Akan. Besides these, similar algal materials have been cultured in various research and exhibition institutions; nevertheless, the same phenomenon has not been observed (Yamada and Sakai, 1961; Yoshida, 1962; Wakana, 2009). Accordingly, the perspective that zoosporogenesis is an extremely rare event has been accepted, or the necessity for reinvestigation has been emphasized (Fritsch, 1961; van den Hoek, 1963; Leliaert and Boedeker, 2007). On the other hand, using natural-epilithic filaments from Lake Akan, M. Satake and K. Ueda (personal communication) observed a small number of zoospores with two or four flagella in May 2001. These findings along with our results indicated that *A. linnaei* has zoospore-forming ability even though its percentage was extremely low regardless of cultured or natural materials.

The rare zoosporogenesis in *A. linnaei* has been thought to be an argument for why its natural population persists by vegetative growth (Fritsch, 1961; van den Hoek, 1963; Kurogi, 1980; Boedeker et al., 2010b). Conversely, Nishimura and Kanno (1927) pointed out a possibility that there is a reproductive process through zoospores based on the existence of the epilithic filaments in Lake Akan. I. Wakana (unpublished results) has been in charge of surveys at Lake Akan since 1991, and has frequently witnessed the examples in which *A. linnaei* filaments grew densely on the surface of artificial substrates, such as a rope, a stake, etc., several years after their installation. In addition, it has been observed that natural-quadriflagellate zoospores develop into juveniles after adhesion under a culture (M. Satake and K. Ueda, personal communication; I. Wakana, unpublished results). These results suggested that discharged zoospores contribute to the persistence of *A. linnaei* populations and to dispersion of its distribution in the field.

Rare zoosporogenesis also appears to contribute to maintaining the aggregative form, which is a specific characteristic of *A. linnaei* through the continuation of vegetative growth over a long period of time. In the Cladophorales, zoospores are formed by differentiation of somatic cells. The mother cells become empty after zoospores are discharged, and the body structure is finally lost. The same phenomenon is also verified in *A. linnai* (Nishimura and Kanno, 1927; Yabu, 1975), and frequent formation and discharge of zoospores in the spherical aggregations would bring about fragmentation. In other words, filaments with low ability of zoosporogenesis can form large spherical aggregations and sustain them in the long term. As if supporting this view, no zoospores were observed in the aggregations from Kinetanpe, and the percentages of zoosporogenesis in the aggregations from Churui were similar or slightly lower than that of epilithic filaments from Jyagaiwa as well as Shurikomabetsu (Table 1), even though a statistically significant difference was not shown. As for such differences in characteristics among the life forms in *A.*

*linnaei*, Soejima et al. (2009) analyzed isozymes of ten populations in Lake Akan including the same populations as the present study, and they found that the aggregative and free-floating forms were genetically monomorphic as opposed to the epilithic form, which showed polymorphism. This result suggested that the aggregative and free-floating forms differ from the epilithic form genetically and persist in their populations by clonal growth. These genetic differences and growth properties seem to fit with differences in the percentages of zoosporogenesis among the life forms. As known in other Cladophorales, external stimuli such as changing in light, temperature, or water quality may induce zoosporogenesis (Lobban and Harrison, 1994), and it will be able to verify with a transplantation experiment in a further study.

In conclusion, we verified the formation of quadriflagellate zoospores in *A. linnaei* in Lake Akan. Although the percentages of zoosporogenesis were extremely low, the discharged zoospores were thought to contribute to the persistence of *A. linnaei* populations. Furthermore, the low percentages of zoosporogenesis appeared to maintain the aggregative form of this alga. However, the biflagellates reported in previous studies (Nishimura and Kanno 1927; Yabu 1975) were not observed. The existence of biflagellate as well as quadriflagellate zoospores implied a possibility that *A. linnaei* has an isomorphous alternation-of-generations type life cycle, like many species of Cladophorales (Bold and Wynne, 1985). Accordingly, rediscovering the biflagellate zoospores and clarifying their role in a life cycle including quadriflagellate zoospores will be important. Although understanding growth and reproduction inclusive of the life cycle of *A. linnaei* has been remarkably difficult in the past because of the rarity of zoosporogenesis, we suggest that the present study effects a breakthrough with these problems.

**Acknowledgements**

We are grateful to Dr. Y. Oyama, Kushiro City Board of Education, Japan, for supporting this research. We also thank Dr. Á. Einarsson, Mývatn Research Station, Iceland, for kind reading of the manuscript, Dr. C. Nagasato, Hokkaido University, Japan, for valuable advice, and Mr. Y. Tsuzuki, Hokkaido University, Japan, for supporting statistical analysis. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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**Table**

Table 1

Percentage of zoospore-producing filaments of *Aegagropila linnaei* in Lake Akan. Six samples were collected per study site, and fifty filaments per sample (in total 300 filaments per site) were isolated. Statistics indicates mean of the six samples ± standard deviation.

| Site | Percentage of zoospore-producing filaments (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Late-May | | Early-August | | Mid-August | | Early-September | | Late-September | | Late-October | |
| | 2017 | 2018 | 2017 | 2018 | 2017 | 2018 | 2017 | 2018 | 2017 | 2018 | 2017 | 2018 |
| Churui | null | null | null | null | null | 0.3±0.7 | 0.3±0.7 | null | null | null | null | null |
| Kinetanpe | null | null | null | null | null | null | null | null | null | null | null | null |
| Takiguchi | null | null | null | null | null | null | null | null | null | null | null | null |
| Jyagaiwa | null | null | null | null | 1.3±1.9 | 0.7±0.9 | 0.7±1.5 | 0.3±0.7 | null | null | null | null |
| Shurikomabetsu | null | null | null | null | 1.0±1.5 | 1.0±1.0 | 0.7±1.5 | null | null | 0.3±0.7 | 0.3±0.7 | null |

**Figure**

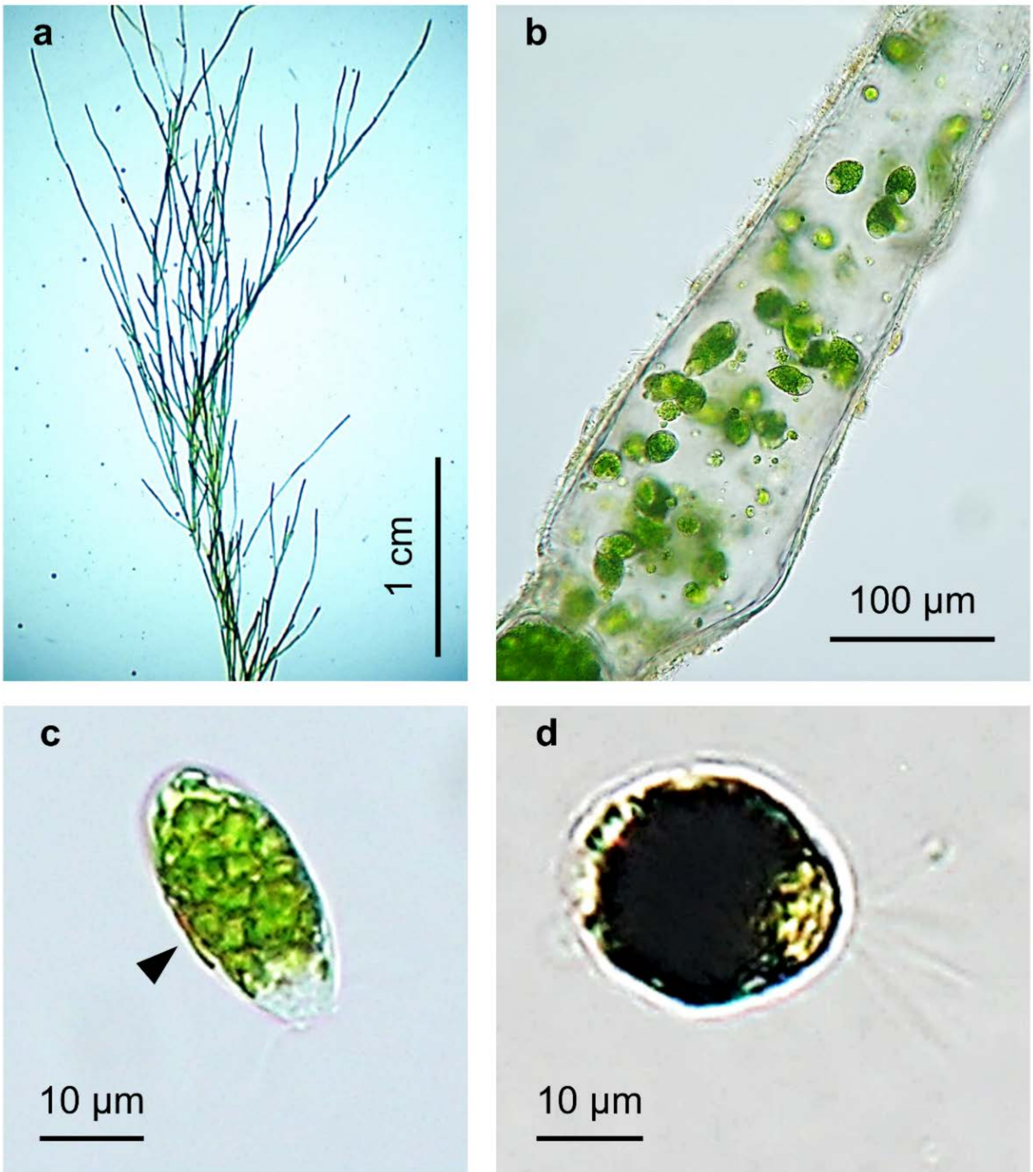


Fig. 1. (a) A filament of *Aegagropila linnaei* isolated from an aggregation of Churui. (b) Zoospores swimming actively within a cell of a filament from Churui. (c) Discharged zoospore with an eye-spot (arrowhead). (d) Zoospore with four flagella after fixation in Lugol's solution.