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Encystment by the mixotrophic flagellate *Ochromonas pinguis* Conrad (Chrysophyceae) under manipulated laboratory conditions

Dale A. Holen[1*] & Sarah Princiotta[2]

[1]*Pennsylvania State University – Scranton, 120 Ridge View Drive, Dunmore, Pennsylvania 18512; *Corresponding author e–mail: dah13@psu.edu*
[2]*Pennsylvania State University – Schuylkill, 200 University Drive, Schuylkill Haven, Pennsylvania 17972*

Abstract: Chrysophyte stomatocysts are well documented in lake sediments, but except for a few studies on sexually reproducing colonial chrysophytes, little is known regarding encystment cues in these algae. *Ochromonas pinguis* Conrad, a mixotrophic chrysophyte with an unknown sexual history was observed to produce stomatocysts, albeit at a low frequency. To determine if the low rate of encystment was typical or whether manipulating environmental conditions might enhance the rate of resting cyst production *O. pinguis* was subjected to various physical and chemical conditions in a batch culture. *O. pinguis* cultures were manipulated by culturing them in DY–V inorganic medium in variable light, pH, and temperature conditions, and at varying levels of N and P limitation. Except for growth in continuous darkness, all treatments resulted in an increase in *O. pinguis* concentration. Five treatments resulted in a corresponding increase in stomatocyst concentration. In the remaining treatments stomatocyst production was steady although sporadic. The encystment frequency however was minimal in all manipulations and ranged from 0.008 to 0.03% of the population undergoing cyst formation. In this study, *O. pinguis* stomatocyst production was not influenced by laboratory manipulations but rather a minute proportion of the population produced stomatocysts regardless of environmental conditions.

Key words: chrysophyte, cyst, dormancy, encystment, mixotrophy, *Ochromonas,* resting stage*,* stomatocyst

Introduction

The development of resting cysts is a life history component of many planktonic microalgae. During periods of environmental stress when conditions become less favorable for growth, vegetative cell encystment increases the likelihood of survival and provides a benthic recruitment pool when conditions improve (Fryxell 1983). The high abundance of resting cysts collected from within the water column and sediments illustrates the significance of this life history trait in the ecology of many algal groups, and underscores this important link between the vegetative pelagic community and the benthic "seed bank" pool (Heiskanen 1993; Belmonte et al. 1995; Stoecker et al. 1998; Firsova et al. 2008). Additionally, encystment provides a mechanism for the short– or long–distant dispersal of algae via waterfowl and other animals (Schlichting 1960). Little, however, is known relative to the development of cysts in many algal groups and under which environmental conditions they are produced.

Although predation (Rengefors et al. 1998) and allelopathy (Fistarol et al. 2004) have been reported to stimulate cyst formation in dinoflagellates, nutrient limitation is often the implicated trigger for algal cyst development (Heaney & Talling 1980; Hargraves & French 1983). Specific environmentally induced cues for encystment however are often difficult to resolve. Moreover, algal cysts can be produced asexually and/or sexually by many taxa and the induction triggers vary. Resolving what induces cyst formation in lakes is problematic as sampling periods are relatively short, even a single sampling date, such that inducement cannot be directly assessed. Agberti & Smol (1995) reported chrysophyte encystment in two Canadian lakes during nitrogen limitation but as it coincided with an increase in temperature and illumination the specific inducement cue could not be determined. And Sheath et al. (1975) suggested rising temperature may be the stimulus for encystment in *Dinobryon divergens* O.E. Imhof but couldn't discount the effects of competition as peak cyst production occurred when phytoplankton concentration and diversity were at a maximum. As such, cause and effect relationships are often inferred as Heiskanen (1993) reported encystment in the dinoflagellate *Peridinium hangoei* Schiller at the end of a spring algal bloom when nitrate was depleted.

Many encystment studies have been laboratory–based where physical and chemical variables can be controlled to assess their individual or cumulative effects on individual species. When grown on a chemically defined medium in batch culture, limiting N and P was the primary factor responsible for encystment in the freshwater dinoflagellate *Peridinium cunningtonii* Lemmermann (Sako et al. 1985), and nitrogen limitation was responsible for encystment in the dinoflagellates *Peridinium cinctum* Müller and *P. willei* Huit.–Kaas with irradiance levels influencing yield (Chapman & Pfiester 1995).

An underlying trait of the Chrysophyceae is their ability to produce stomatocysts, a more specific term for the siliceous resting cysts in these algae (Kristiensen & Preisig 2001). Vegetative cells which are haploid divide by cell division and under certain conditions produce stomatocysts asexually. Sexual reproduction can also result in stomatocyst formation by the fusion of compatible mating types to form a planozygote which undergoes encystment (Sandgren 1991). As chrysophytes distribution patterns have been well defined along environmental gradients, stomatocysts in lake sediments can be used as paleolimnological markers to reconstruct past environmental changes (Duff & Smol 1994; Huber et al. 2009), yet few stomatocysts have been linked to their vegetative counterpart. Less is known regarding the conditions under which stomatocysts develop as few inducement studies have been undertaken with chrysophytes and their life histories have not been well documented. As chrysophyte algae are often a dominant component of the phytoplankton in oligotrophic lakes (Sandgren 1988), identifying the factors involved in the development of their resting cysts is important in understanding their reproductive strategies and in identifying underlying environmental factors involved in their seasonal population periodicities.

Ochromonas are a polyphyletic assemblage of bi–flagellate chrysophytes with over 125 species, both freshwater and marine (Andersen et al. 1999; Andersen 2007). They are primarily mixotrophic exhibiting a broad range of nutritional capabilities from predominately phototrophic to predominately phagotrophic (Holen & Boraas 1995). Their sexual history is unknown. *Ochromonas pinguis* Conrad, a large and predominately phototrophic flagellate (Holen 2010), was observed to produce stomatocysts when cultured on DY–V medium at 25 °C on a 16:8 light/dark cycle at 100 $\mu mol.m^{-2}.s^{-1}$ but very few were detected (Holen 2014). As there are few laboratory studies of encystment in chrysophytes, and none with *Ochromonas* genera, the intent of this investigation was to subject *O. pinguis* to various stimuli; daylength, temperature, pH, and nutrient limitation in batch culture to determine if they influence encystment rate. Despite the influence of physical and chemical factors on cyst induction reported for some alga taxa and because of the reports by Sandgren (1981) and Sandgren & Flanagin (1986) with asexual populations of *Dinobryon* and *Synura*, it is predicted that stomatocyst production in this clonal population of *O. pinguis* will not be influenced by the extrinsic forces.

Materials and Methods

The chrysophyte used in this study was *Ochromonas pinguis*, a mixotrophic flagellate isolated from Lake Lacawac (41°22'54.5" N, 75°17'34.3" W), a mesotrophic lake located in the Lacawac Sanctuary, an environmental education center and biological field station in Northeast Pennsylvania. For descriptions of the isolation procedure and the flagellate's mixotrophic capabilities refer to Holen (2010). The flagellate culture was not axenic as repeated attempts to produce a bacterial–free culture using antibiotics (Stein 1973) proved unsuccessful. In cultures where bacteria appeared to be absent the flagellate population did not survive suggesting this chrysophyte may have an obligate requirement for particulate food (Holen 2010). For a morphological description of the stomatocyst refer to Holen (2014).

Between trials the flagellate was maintained in DY–V inorganic growth medium (Andersen et al. 2005), pH 6.8 at 20 °C in a temperature–controlled incubator. Illumination at approximately 150 $\mu mol.m^{-2}.s^{-1}$ (PAR) was provided by a bank of cool–white, fluorescent bulbs mounted on the door of the incubator and set on a 12:12 light/dark regime. Stock cultures for all trials were maintained in this manner for a period of approximately three to four weeks when the population reached a stationary phase of growth. To minimize carry–over of nutrients from the stock culture to the experimental flasks centrifugation of flagellates was attempted but unsuccessful due to high mortality, presumably because of the lack of a cell wall. Instead, a minimal inoculum (approximately 2 ml) of the stock culture was transferred to 150 ml of sterile–filtered DY–V (replete or modified) which was then divided equally between three sterile, glass Erlenmeyer flasks to initiate each experimental run. All experimental flasks were initially washed with Micro–90 cleaning solution (International Products, Corp., Burlington, NJ), rinsed with 10% HCl, and then repeatedly rinsed with distilled water.

All trials were run in triplicate and unless otherwise noted were performed in a temperature–controlled, illuminated incubator on a 12:12 hour light/dark cycle at 150 $\mu mol.m^{-2}.s^{-1}$ at 20 °C. Dark trials were run in aluminum foil–wrapped flasks. The treatments included culturing *O. pinguis* in replete DY–V a) in the light, b) in the light with added bacteria, c) in the dark, d) at 10 °C, e) at pH 5.8, and f) at an 8:16 light/dark regime. As Lake Lacawac has a pH of 6.4 (Pilla et al. 2017) the pH of DY–V in that treatment was reduced to 5.8 to represent a moderate increase in acidity. To simulate nutrient limitation *O. pinguis* was cultured in the incubator as stated above but in modified DY–V. Moderate nutrient limitation treatments included G) 1% N and H) 10% P. Extreme nutrient limitation treatments included I) 0% N & P and J) 0% N & P at 10 °C.

In the light trials with added bacteria, the bacterium (≈ 1.0 µm bacillus) was originally isolated from Lake Lacawac. The isolate was grown on Tryptic Soy Agar (Difco, Becton, Dickinson and Company, New Jersey) for 48 hours, scrapped off the plate, added to a test tube containing DY–V, and then vortexed to suspend. The bacteria were added to the aluminum foil–capped experimental flasks containing replete DY–V at a

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starting concentration of approximately 4.7×10^6 bacteria.ml^{-1}. Samples were collected every 48 hours over a 25–day period to enumerate flagellates and stomatocysts. Each flask was gently shaken from which one– or two–ml samples were withdrawn, fixed with glutaraldehyde (4% final concentration), stained with DAPI as a fluorochrome (2.5 µg.ml^{-1} final concentration) for six minutes, filtered onto black 25 mm, 0.2 µM pore–size polycarbonate filters (Whatman International LTD., England), and mounted onto slides (Hobbie et al. 1977). To enumerate flagellates 40 fields per filter were counted at 1000× using an Olympus BX51 microscope fitted with a X100 UPlan Apo objective lens (Olympus Corp., Tokyo, Japan) and using UV irradiation. For the enumeration of stomatocysts the entire filter was scanned using a X10 UPlanFLN objective lens. The filter was scanned in overlapping rows until the entire filter was counted. Stomatocysts that were observed were scored as either active or inactive. Active stomatocysts contained a viable cell as evidenced by chlorophyll autofluorescence when illuminated with blue light (460–495 nm, BP490 filter cube). Inactive stomatocysts were empty and included in that count to assure that all stomatocysts, regardless of the timing of excystment, were enumerated. Encystment percentage (cyst.ml^{-1}/flagellate.ml^{-1} × 100) were determined at each time interval to determine the maximum frequency of stomatocyst production. Only the maximum percentage for each manipulation is given. Growth rates were calculated from linear regressions of log–transformed *O. pinguis* concentration vs time when the increase was exponential.

Individual, one–way ANOVA tests were used to examine the effect of treatment on growth rate or rate of cyst formation. As there was a significant effect of treatment, a post–hoc Tukey HSD test revealed between which treatments statistically significant differences existed. Analyses were conducted in R (R Core Team, 2012).

Results

In all manipulations including nutrient deficient, the stock culture of *O. pinguis* was not axenic and it is thus likely that the alga obtained some nutrition through the ingestion of residual bacteria in the medium. However, given that the growth rates of *O. pinguis* were lower when subjected to nutrient limitation, in comparison to growth on replete DY–V, suggests that bacterivory did not contribute significantly as a dietary supplement (Table 1). Furthermore, microscopic examination of test cultures indicated that bacterial numbers were less than 1×10^6 cells.ml^{-1}, a threshold concentration consistent with the stationary phase of flagellate growth.

There was a significant effect of treatment on growth rate ($p < 0.001$, $F_{7,16} = 14.84$). There were no significant differences between *O. pinguis* growth rate when cultured in DY–V in the light, in DY–V with added bacteria, and 10% P treatments. However, these treatments are significantly different from pH 5.8, 10 °C, 0% N & P, and 0% N & P + 10 °C treatments.

Encystment by *O. pinguis* was observed in all manipulations except when the flagellate was cultured in the dark although the encystment rate never exceeded, on average, four stomatocysts.ml^{-1} at any sampling period regardless of manipulation or flagellate concentration. Moreover, stomatocyst concentration was highly variable within and between sampling periods. An increase in *O. pinguis* concentration resulted in a corresponding increase in stomatocysts in the DY–V in the light, DY–V in the light with added bacteria, pH of 5.8, 1% N, and 10% P treatments. For the remaining treatments stomatocyst production remained relatively consistent although sporadic at times as the flagellate population increased. There is a significant effect of treatment on *O. pinguis* cyst formation over time ($p = 0.02$, $F_{7,16} = 3.51$). A post–hoc Tukey LSD test revealed significant differences in rate of cyst formation that occurred only between the DY–V in the light with added bacteria and 0% N & P + 10 °C treatment ($p < 0.05$), with higher rates in the treatment with bacteria.

When cultured in the light on replete DY–V the mean stomatocyst production was relatively stable at less than 1.0 cysts.ml^{-1} per sampling period but then increased to a maximum of 1.4 cysts.ml^{-1} (Fig. 1A). The highest percentage of flagellates producing cysts was 0.009%. With bacteria added as a food source the *O. pinguis* concentration increased for 12 days before declining and then increasing once again. The mean cyst production increased to a maximum of 4.0 cysts.ml^{-1}; the increase coinciding a decline in the flagellate population with a maximum of 0.01% of the population produced stomatocysts (Fig. 1B). There was no growth of *O. pinguis* when cultured in DY–V in the dark. The flagellate population declined steadily and no stomatocysts were observed over 15 days (Fig. 1C). When cultured on replete DY–V in the light but at a reduced temperature of 10 °C the *O. pinguis* concentration increased gradually over 30 days. During this

Table 1. *O. pinguis* population growth rates ± standard deviation for all treatments. Hourly growth rates were calculated from the linear portion of the log–transformed *O. pinguis* concentration over time.

Treatment	**µ.h^{-1}**
DY–V (light)	0.016 (0.002)
DY–V (dark)	0
DY–V + added bacteria	0.018 (0.003)
pH 5.8	0.011 (0.002)
8:16 h light/dark cycle	0.012 (0.001)
10 °C	0.009 (0.002)
1% N	0.012 (0.002)
10% P	0.015 (0.003)
0% N & P	0.007 (0.002)
0% N & P + 10 °C	0.004 (0.0006)

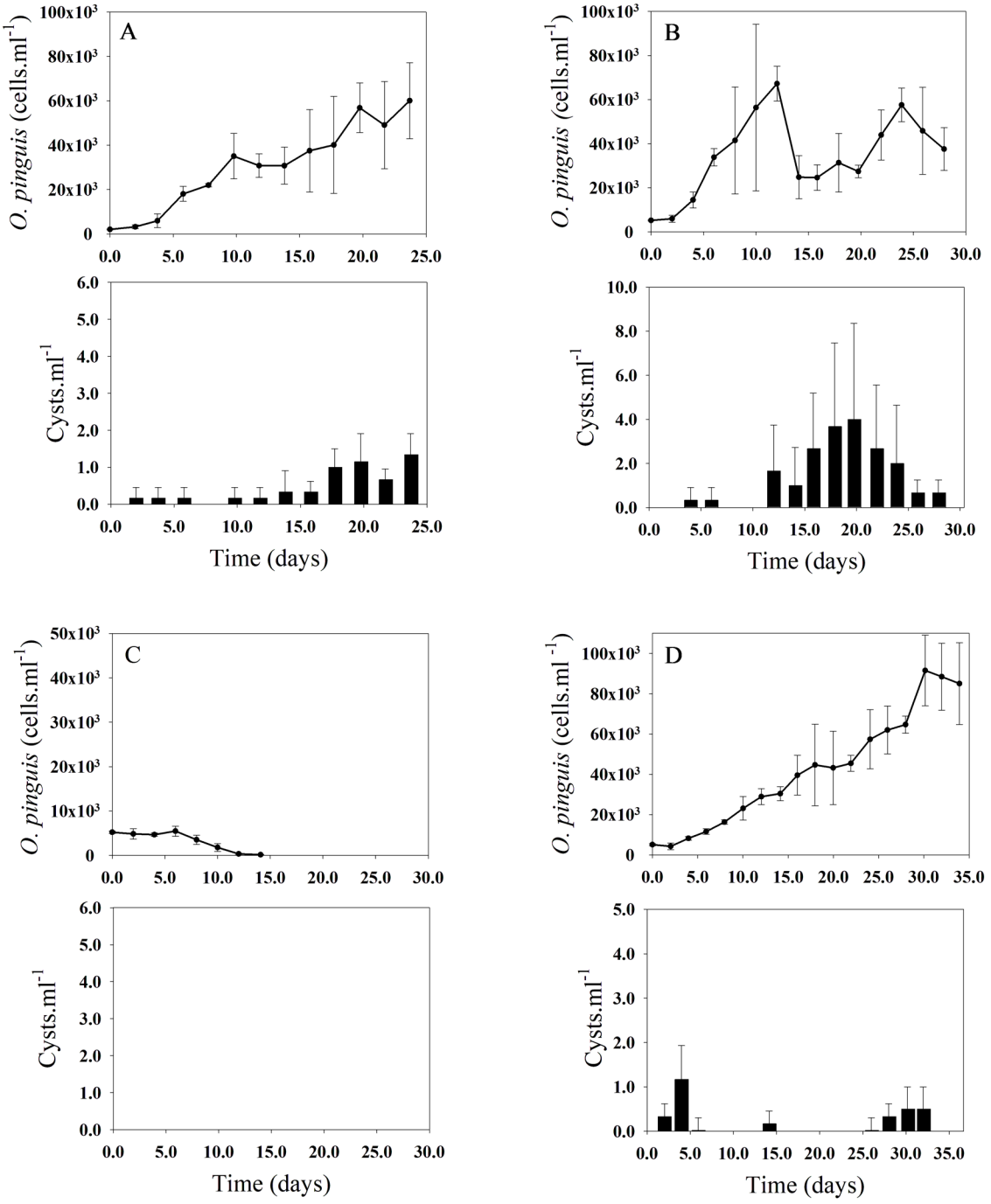

Fig. 1. Cell concentration of *O. pinguis* (top) and stomatocyst production (bottom). Growth in replete DY–V: (A) at a 12:12 light/dark cycle without added bacteria, (B) with added bacteria, (C) in the dark, (D) at a reduced temperature of 10 °C. Error bars represent ± 1 standard deviation.

interval stomatocyst production was sporadic and generally below 1 stomatocyst.ml^{-1} with a maximum of 0.015% of the population producing cysts at four days (Fig. 1D). At a reduced pH of 5.8 stomatocysts were produced throughout the study with a maximum of 3.0 stomatocysts.ml^{-1} occurring at approximately 19 days with a maximum of 0.012% of the population encysting (Fig. 1E). Given a light/dark exposure cycle of 8:16 hours the flagellate population increased steadily for 27 days with a stomatocyst production rate that was intermittent and on average of less than 1.0 stomatocyst.ml^{-1} (Fig. 1F.). The percentage of flagellates producing stomatocysts never exceeded 0.008%. Because of an incubator malfunction resulting in the loss of the *O. pinguis* culture, additional temperature manipulations were not possible.

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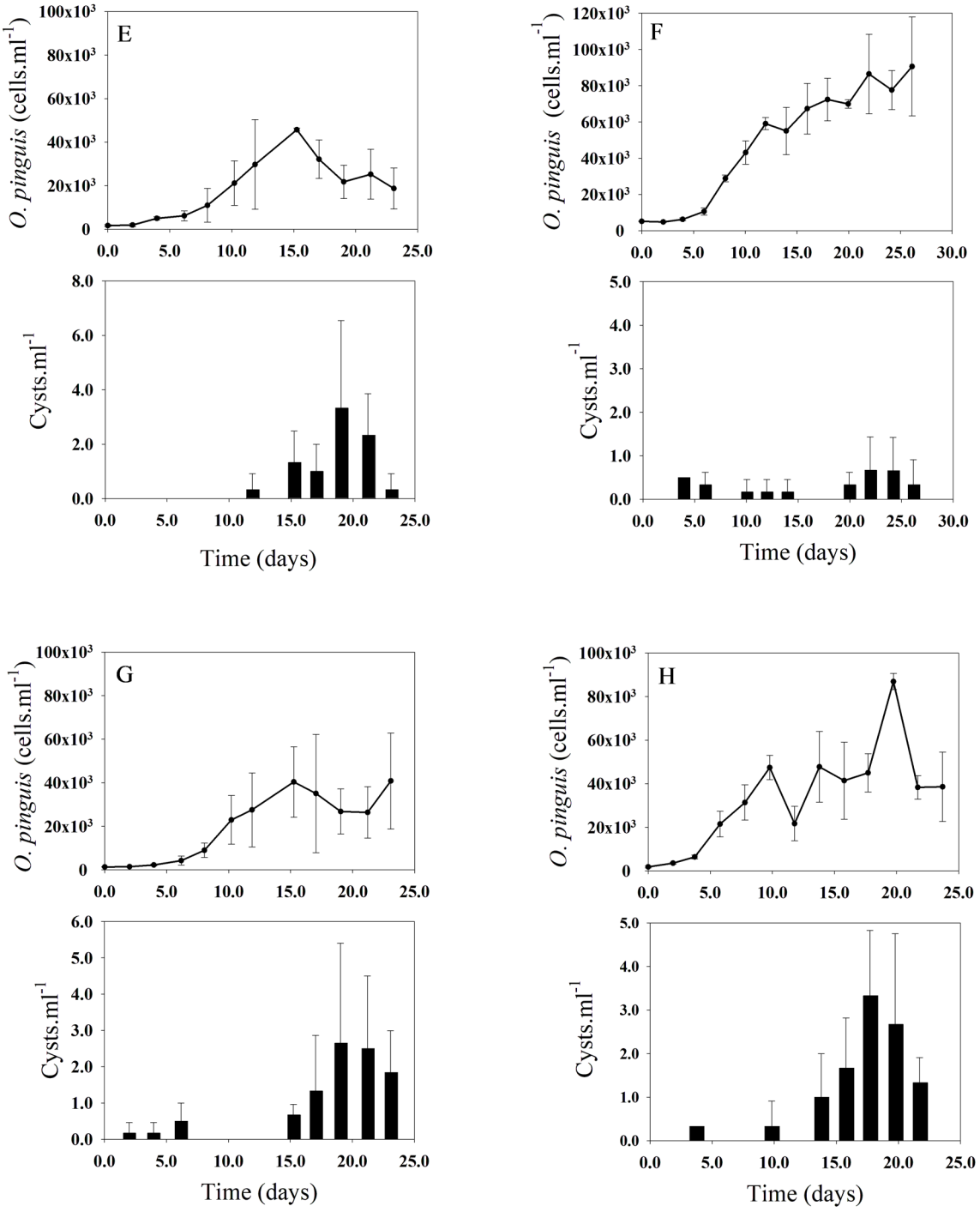

Fig. 1 (continued). Cell concentration of *O. pinguis* (top) and stomatocyst production (bottom). Growth in replete DY–V: (E) at a reduced pH of 5.8; (F) growth in replete DY–V on an 8:16 light/dark cycle. Growth in modified DY–V: (G) 1% N, (H) 10% P. Error bars represent ± 1 standard deviation.

In DY–V treatments with 1% N, stomatocyst production was minimal in the beginning before ceasing (or undetectable) between eight and 14 days. As the flagellate population declined at 16 days stomatocyst production was observed to increase to a maximum of 0.03% of the population producing cysts (Fig. 1G). When the DY–V medium contained 10% P the majority of the stomatocysts were produced after 13 days when the population density leveled except for a peak at 20 days (Fig. 1H). A maximum of 3.3 stomatocysts.ml^{-1} were produced at 18 days before declining with less than 0.007% of the population producing stomatocysts at any time.

When subjected to DY–V in the light with 0% N and P the flagellate population increased slowly but

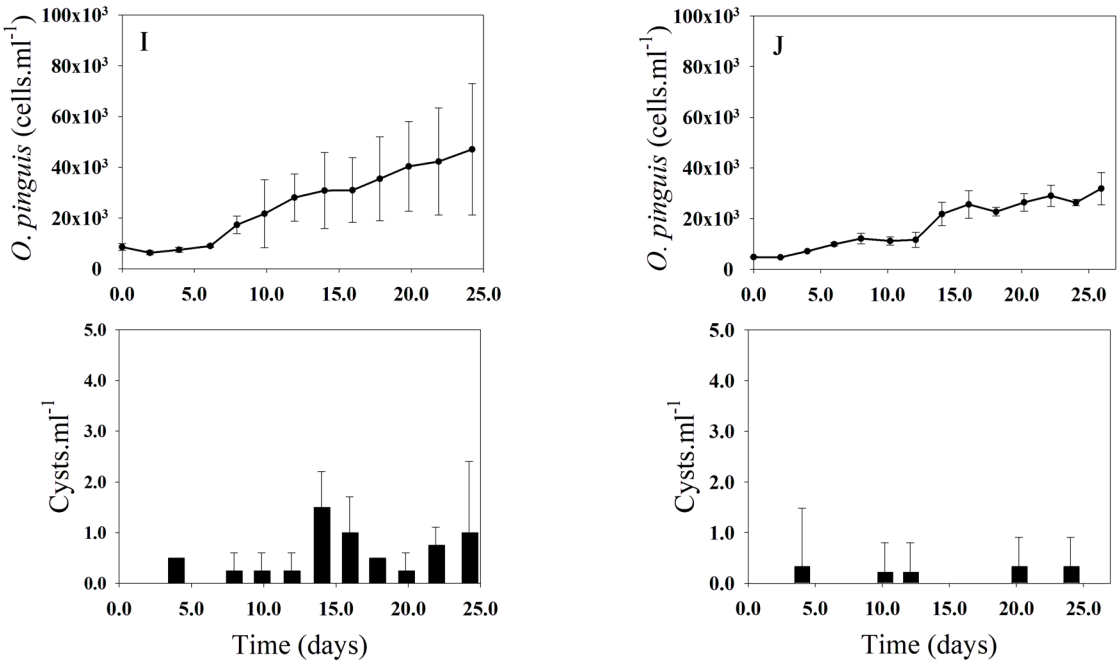

Fig. 1 (continued). Cell concentration of *O. pinguis* (top) and stomatocyst production (bottom) in modified DY–V. Growth in: (I) 0% N & P and (J) 0% N & P at 10 °C. Error bars represent ± 1 standard deviation.

steadily. The mean stomatocyst production was less than 1.0 ml^{-1} for the first 13 days, increasing to 1.5 ml^{-1} at 14 days before decreasing. A maximum of 0.007% of the population produced stomatocysts. (Fig. 1I). *Ochromonas pinguis* exhibited its lowest growth rate when cultured in DY–V in the light with 0% N and P and 10 °C. The stomatocyst production was intermittent but steady at less than 1.0 stomatocyst.ml^{-1} with stomatocyst production never surpassing 0.009% of the population (Fig. 1J).

Discussion

There are no published reports in the literature where multiple physical and chemical variables were tested to determine their influence on encystment in asexual chrysophyte species. In this study *O. pinguis* was exposed to laboratory conditions of nutrient limitation, light limitation, bacterial prey, pH, and temperature to determine their individual or cumulative effect on encystment. *O. pinguis* was exposed to both moderate and extreme nutrient limitation as some algae have been shown to produce cysts only when nutrients are limited but not absent (Chen et al. 2015). As it was hypothesized, cyst formation by *O. pinguis* did not respond to environmentally induced stress with increasing encystment but rather a minute percentage of the population encysts regularly despite the environment conditions.

These observations are consistent with clonal populations of the chrysophyte *Dinobryon cylindricum* O.E. Imhof where the encystment rate under nutrient limitation ranged from 0 to 0.05% (Sandgren 1981). Likewise, rates of stomatocyst production in *Synura petersonii* Korshikov ranged from 0.001 to 0.01 cysts. cell^{-1} when nutrient deficient (Sandgren & Flanagin 1986). However, Yubuki et al. (2008) reported that *Spumella* sp., an aplastidic chrysophyte, did not respond to nutrient limitation but, like *D. cylindricum* and *S. petersonii*, stomatocyst induction was density–dependent with a threshold concentration of 4.3 to 5.3×10^4 cells.ml^{-1} for encystment to ensue. In many chrysophyte genera, stomatocyst production has never been observed despite treatment effects. When cultured on an inorganic basal medium and exposed to a range in temperature and light regimes only six of 90 strains of *Spumella* spp. encysted in laboratory culture (Fendenig et al. 2010). Clearly, our knowledge of the life histories of chrysophytes is incomplete and given the paucity of laboratory studies it is possible that the induction cues stimulating stomatocyst formation in *O. pinguis* and other chrysophytes have not been identified.

Cyst production in dinoflagellates appears more diverse with both asexual (temporary) and sexual (resting) cysts produced although more complex variations have been described (Bravo & Figueroa 2014). Like chrysophytes, clonal populations of *Peridinium cinctum* and *P. willei* exhibited low encystment rates ranging from 0 to 4.1% of the population when subjected to physical and chemical stressors (Chapman & Pfiester 1995). However asexual cyst formation may be substantive

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as 10–70% of clonal strains of *Prorocentrum cordatum* Ostenfeld encysted when subjected to centrifugation, decrease in temperature, and increase or decrease in salinity (Matantseva et al. 2020). Likewise, both clonal, and mixed–strain cultures of *Scrippsiella hangoei* Schiller exhibited high rates of cyst formation when subjected to increasing temperature, but with 95% of the cysts in the mixed–culture treatments being asexually produced (Kremp & Parrow 2006).

Binucleate stomatocysts were observed in this study but sexual reproduction in *Ochromonas* is undetermined and cell fusion was not observed. Andersen (1982) reported stomatocyst production by *O. sphaerocystis* Matvienko when in culture, but cell fusion nor binucleate stomatocysts were observed so they were assumed to be asexually produced. However, Andersen et al. (2017) reported stomatocyst formation in mixed cultures of *O. triangulata* Vysotskii when subjected to cold temperatures which suggests heterothallic sexual reproduction as clonal populations subjected to the same temperature conditions did not encyst (personal communication). Sandgren (1981) suggested that the binucleate stomatocysts observed in clonal populations of *Dinobryon cylindricum* are the result of autogamy, a pre–encystment nuclear division. This has also been reported by Dangeard (1910) and Gayral et al. (1972) for other chrysophytes as well as in diatoms (Reddy 2001). The reproductive significance of this mode of reproduction is not clear. Alternatively, the binucleate stomatocysts in this study could be the result of homothallism, the fusion of clonal cells prior to stomatocyst development as has also been reported in the loricate chrysophyte *Kephyrion* (Kristiansen 1963). Homothallism has been well documented in other algal groups, including species with both homothallic and heterothallic strains, e.g., dinoflagellates (Destombe & Cembella 1990, Blackburn et al. 2001, Figuero et al. 2010), green algae (Tsuchikane et al. 2012), and diatoms (Montresor et al. 2016).

Additionally, on occasion, extremely large *O. pinguis* unicells with multiple long and short flagella were observed in this study. Similar giant cells of an Antarctic strain of *Ochromonas minuscula* Conrad have also been reported by Curtin (1987). The significance of these large, multi–flagellated and multi–nucleated cells is unknown, nor is their origin. Are they the result of cell divisions minus cytokinesis or multiple cell fusions?

In fixed samples, there was considerable morphological variation in stomatocysts which appeared to be differing degrees of cyst wall development. Most stomatocysts appeared fully developed but the outer cyst wall of some stomatocysts exhibited a paucity of ridges all of which were undersized. Sandgren (1981) also reported incomplete development in stomatocysts of *D. cylindricum*. The under–developed stomatocysts in this study may be the result of arrested development because of sample collection and fixation. However, some of these stomatocysts were free of vegetative cells suggesting excystment had ensued and implies a natural variation in cyst morphology. This is supported by Siver (1991) who observed varying degrees of stomatocyst development in *Mallomonas acaroides* Perty emend. Ivanov from core samples taken from Little Echo Pond in New York, U.S.A.

Encystment is a survival strategy in chrysophytes but is also linked to sexual reproduction. Growth profiles of genera, e.g., *Dinobryon, Uroglena, Mallomonas* reveal sudden and rapid appearances in lakes followed by a rapid decline (Sandgren 1988). This seasonal spike in population density increases the likelihood of cell contact by compatible mating types and can produce a large refuge population of cysts which descend to the sediments. Species of *Ochromonas* in contrast produce stomatocysts at a low but steady frequency if this study is indicative of *Ochromonas* biology. They are cosmopolitan in their distribution, found in a variety of aquatic habitats including acidic lakes (Albertano et al. 1994) and ice and snow of Alpine lakes (Felipe et al. 1995) and have been reported to be present year–round in lakes in which they may become dominant (Bennett et al. 1990; Tittel et al. 2003). Thus, their contribution to the sediment seed bank is most likely substantial.

Encystment frequencies in natural chrysophyte populations most likely differ with encystment being initiated by individual or multiple environmental factors. The pattern of *O. pinguis* encystment is of low but consistent stomatocyst formation regardless of exogenous influence. To determine if these findings accurately represent encystment dynamics in asexual chrysophytes and to acquire a more complete understanding of their reproductive biology additional detailed experimental studies are needed on individual species.

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