Journal of Oceanology and Limnology
Vol. 36 No. 2, P. 273-280, 2018
https://doi.org/10.1007/s00343-018-6291-x

Solid sand particle addition can enhance the production of resting cysts in dinoflagellates*

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Received Oct. 26, 2016; accepted in principle Dec. 18, 2016; accepted for publication Feb. 10, 2017

Abstract Resting cysts are an important part of the life cycle for many harmful algal bloom-forming dinoflagellates, and play vital roles in the recurrence and geographical spread of harmful algal blooms. Numerous factors have been suggested to regulate the formation of resting cysts, although only a few have been proven to be significant. Cyst formation can be induced by adverse environmental conditions such as drastic changes in temperature, light, salinity, and nutrient levels, and by biological interactions. In this study, we evaluated the ability of an artificial factor (fine sand particles) to enhance the formation of resting cysts. Fine sand particles were added to cultures of dinoflagellates that are known to produce cysts. The addition of fine sand particles significantly increased both the production rate and final yield of cysts in cultures of *Scrippsiella trochoidea*, *Biecheleria brevisulcata*, and *Levanderina fissa* (=*Gymnodinium fissum*, *Gyrodinium instriatum*, *Gyrodinium uncatenum*). The largest increase in the final yield (107-fold) of cysts as a result of sand addition was in *S. trochoidea*. However, addition of fine sand particles did not induce cyst formation, or barely affected cyst formation, in *Akashiwo sanguinea*, *Cochlodinium polykrikoides* and *Pheopolykrikos hartmannii*, which are also known to be cyst-producing species. We speculated that addition of sand significantly increased the chances of cell collision, which triggered cyst formation. However, further research is required to test this idea. Importantly, our findings indicate that the addition of fine sand particles is a useful method to obtain a large quantity of cysts in a short time for laboratory studies or tests; for example, if a cyst viability test is being used to assess the effectiveness of ships' ballast water treatment.

Keyword: sand; resting cyst; encystment; *Scrippsiella trochoidea*; *Biecheleria brevisulcata*; *Levanderina fissa*

1 INTRODUCTION

Harmful algal blooms (HABs) have been a serious concern worldwide in recent decades, and the main causative organisms of HABs are dinoflagellates (Hallegraeff, 1993; Smayda, 1997; Anderson, 2009). Among about 2300 species of dinoflagellates, approximately 200 species, some of them HAB-forming, produce resting cysts during their life cycle (Bravo and Figueroa, 2014). Resting cysts are an important part of the life cycle of many HAB-forming dinoflagellates, because they play vital roles in resistance to adverse conditions and in the recurrence and geographical spread of HABs (Matsuoka and Fukuyo, 2000; Bravo and Figueroa, 2014; Tang and Gobler, 2015). Resting cysts produced in the laboratory have been used to test the effectiveness of ships' ballast water treatments, because they can survive environmental stresses such as hypoxia, extreme temperatures, darkness, and extreme salinity

* Supported by the NSFC-Shandong Joint Fund for Marine Ecology and Environmental Sciences (No. U1606404), the National Natural Science Foundation of China (Nos. 41476142, 61533011, 41506143), the Scientific and Technological Innovation Project supported by the Qingdao National Laboratory for Marine Science (No. 2016ASKJ02), and the NSFC-Guangdong Joint Fund (No. U1301235)
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Table 1 Origin, date of isolation, and isolator of dinoflagellates used in this study

Species	Strain No.	Origin	Date of isolation	Isolator	Clonal
Scrippsiella trochoidea (Stein) Loeblich III	STBDH1	Beidaihe, Hebei, China	Cyst germination, Aug. 2014	HU Zhangxi	Yes
Biecheleria brevisulcata K. Takahashi & Iwataki	BBNJD2	Nanji Island, Zhejiang, China	Cyst germination, Aug. 2014	HU Zhangxi	Yes
Levanderina fissa (Levander) Moestrup, Hakanen, Hansen, Daugbjerg & Ellegaard	LFJZB1	Jiaozhou Bay, Shandong, China	Vegetative cell, Aug. 2016	HU Zhangxi	Yes
Cochlodinium polykrikoides Margalef	CP1	Flanders Bay, NY, USA	Vegetative cell, Aug. 2006	TANG Yingzhong	Yes
Akashiwo sanguinea (Hirasaki) G. Hansen & Moestrup	CCMA256	Xiamen, Fujian, China	Vegetative cell, Feb. 2011	LUO Zhaohe	Yes
Pheopolykrikos hartmannii (Zimmermann) Matsuoka et Fukuyo	PHJZB2	Jiaozhou Bay, Shandong, China	Vegetative cell, Aug. 2015	HU Zhangxi	Yes

due to their thickened organic walls and their state of physiological dormancy (Hallegraeff and Bolch, 1991).

Because of their significance, dinoflagellate cysts have been the focus of many studies. Previous studies on cysts have focused on their identification and detection, spatial distribution, and morphology (Wall, 1965; Anderson et al., 1988; Qi et al., 1997; Wang et al., 2004; Balkis et al., 2016), their geographical spread via ships' ballast water or shellfish stocks (Hallegraeff and Bolch, 1991; Bolch and de Salas, 2007; Smayda, 2007; Tang and Gobler, 2015), their germination and factors affecting it (Kokinos and Anderson, 1995; Figueroa et al., 2005; Figueroa et al., 2011), and their relationships with the end and recurrence of HABs (Anderson, 1997; Kim and Han, 2000; Kremp and Anderson, 2000; Wang et al., 2007; Bravo and Figueroa, 2014; Mardones et al., 2016).

Other studies have investigated the formation of cysts in the laboratory, and the factors that influence this process. Numerous environmental factors have been shown to affect encystment. Among these factors, nutrient stress such as nitrate and/or phosphate depletion and extreme temperatures are usually regarded as the main factors that affect and induce cyst formation (Anderson et al., 1985; Meier et al., 2004; Nagai et al., 2004; Kremp et al., 2009). For *Scrippsiella trochoidea* (Stein) Loeblich III, a cosmopolitan HAB-forming dinoflagellate, the highest cyst generation ratio in the laboratory was 8.8% (Qi et al., 1997). For *Alexandrium catenella* (=*Alexandrium pacificum*), the highest cyst production in laboratory cultures was obtained under N and P limitation (L/15), and N limitation was more efficient than P limitation in inducing cyst formation (Figueroa et al., 2005). Increased cyst formation has also been observed in response to changes in day length (Sgrosso et al., 2001), parasitic associations (Chambouvet et al., 2011), and salinity and irradiance shocks (Mardones et al., 2016). The results of those studies suggested that the factors regulating cyst formation are more complicated than first thought.

In this study, we report that the addition of solid sand particles significantly enhanced the final yield and production rate of resting cysts in cultures of some dinoflagellates. The methods described in this paper can be used to produce large quantities of resting cysts for use in studies or routine tests.

2 MATERIAL AND METHOD

2.1 Microalgal cultures and experimental conditions

The dinoflagellates used in this study are listed in Table 1. Clonal cultures were established via single-cell isolation using a capillary tube under an inverted microscope, and were maintained in f/2 medium (-Si) (Guillard, 1975) made with autoclaved and sterile filtered (0.22 μm) seawater (salinity 30–31). The identity of each species was confirmed by its 18S and 28S ribosomal DNA sequences. Sand was collected from a beach near the Institute of Oceanology, CAS, and sieved (<100 μm), washed, and sterilized before being added to the cultures at densities of (1.3–1.6)×10^3 kg/m^3.

The experiments were performed in 12-well cell culture plates (Corning 3513, NY, USA). The 12 wells were divided into four groups with triplicate wells for each group. To each well (3.8 cm^2 bottom area) was added 0 (control), 0.01, 0.02, or 0.04 g sand particles after a pre-optimization experiment to balance the light-blocking effect and the convenience of cell counting. The cell density was determined by counting triplicate 1-mL samples fixed with 2% (v/v) Lugol's solution. Penicillin-streptomycin solution (100×, Solarbio, Beijing, China) was added to a final concentration of 2% before inoculation to discourage bacterial growth. The plates were incubated at 21±1°C under a 12-h light/12-h dark photoperiod with

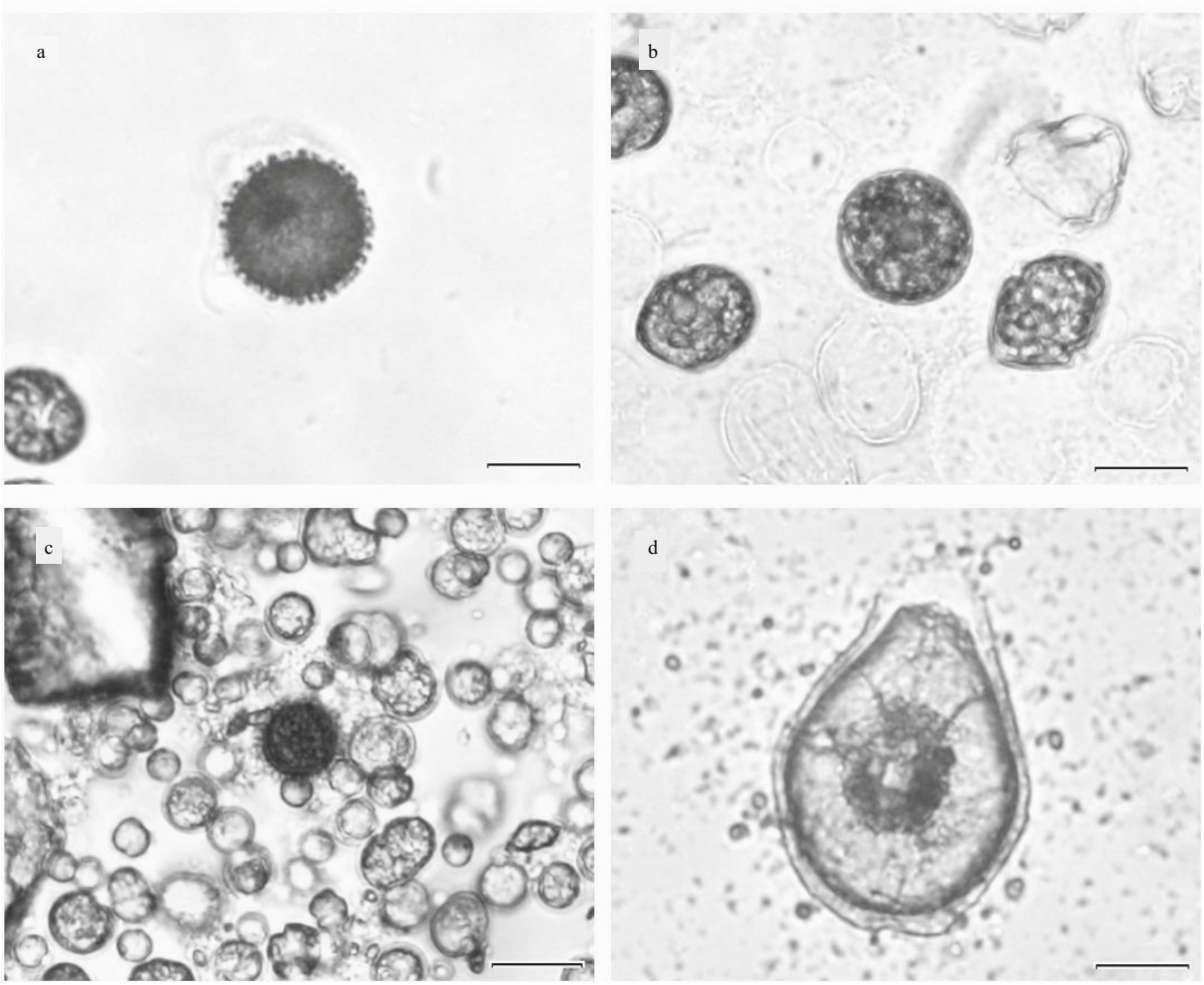

Fig.1 Micrographs of resting cysts

a and b. *Scrippsiella trochoidea* resting cysts with or without calcareous spines, and bright accumulation body which is in red with naked eyes; c. *B. brevisulcata*, with spines, and bright accumulation body which is in red with naked eyes; d. *Levanderina fissa* resting cysts with smooth surface, thick double-layered walls, and accumulation bodies at center. Scale bars=20 μm.

irradiance of 100 μmol photons/(m^2·s) supplied by white fluorescent lights. The cultures were monitored every day.

2.2 Cyst counting and statistical analyses

Resting cysts were counted under an inverted microscope and identified based on their morphological features, namely, the presence of red bodies, absence of chloroplasts, cell wall structure, color, and surface ornamentations such as spines. In general, the resting cysts of all species used in the study were easily identifiable (see Fig.1a–d) (Qi et al., 1997; Gu et al., 2008; Moestrup et al., 2014; Takahashi et al., 2014).

Cysts were counted in a "S" shape from the top to the bottom of each well at 100× magnification under an inverted microscope (IX73, Olympus, Tokyo, Japan) every day or every two days, depending on the cyst production rate in the early stages. Photographs and videos of resting cysts were taken with a digital camera mounted on the microscope (Olympus).

The average production rate of resting cysts ($R_{avg.}$) in each well was defined as follows:

$$R_{avg.}=(Y_{max}-Y_{min})/T,$$

where Y_{max} is the highest number of cysts recorded in a well, Y_{min} is the number of cysts recorded on the first day when cysts were observed, and T is the number of days from Y_{min} to Y_{max} for that well.

The software SPSS Statistics 22 (SPSS, IBM, USA) was used to perform one-way analysis of variance (ANOVA) to test the significance of differences in cyst production rate and cyst yields of each species among the different treatments, with the significance level set to $P<0.05$.

Table 2 Comparison of production rate of resting cysts among control and sand-treated groups (see text for definition of production rate)

Species	Production rate (cysts/d) (mean±SD, *n*=3)			
	Control	0.01 g	0.02 g	0.04 g
S. trochoidea	10.4±5.5[a]	21.6±7.2[a]	137.3±9.4[b]	202.5±15.6[b]
B. brevisulcata	0[a]	53.1±13.7[b]	126.8±31.6[b]	66.8±29.7[b]
L. fissa	209.1±90.7[a]	391.3±75.2[b]	311.0±104.2[ab]	394.5±25.9[b]

The data are expressed as mean±SD; data in the same row with different letter are statistically different (P<0.05).

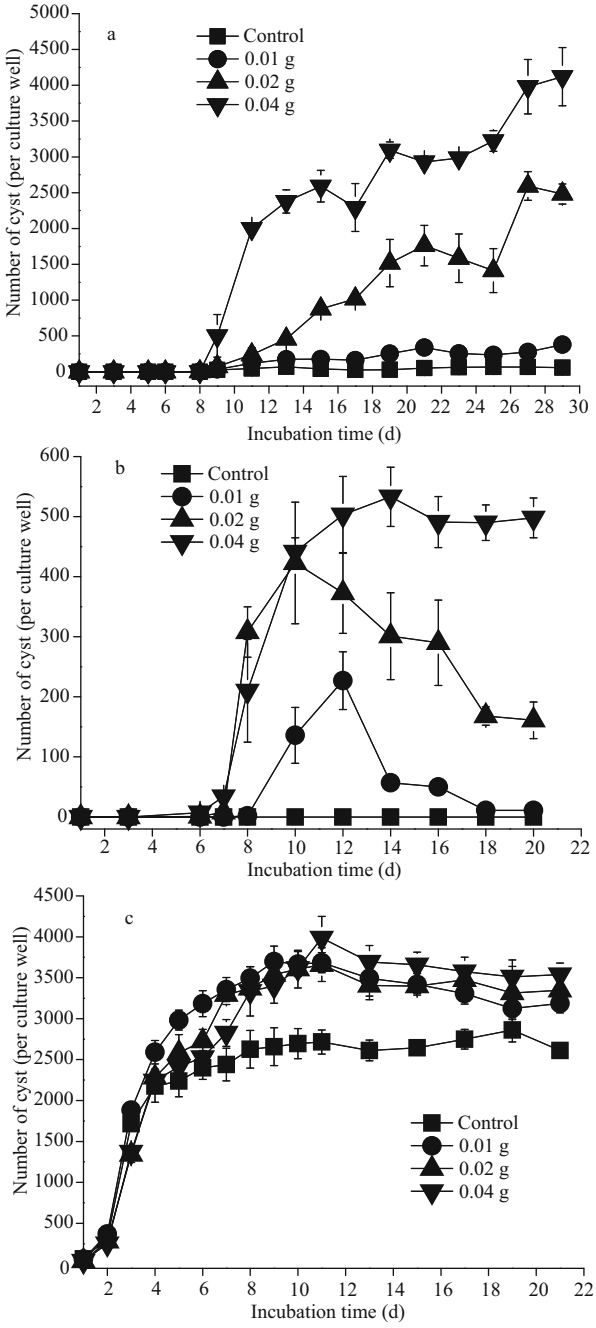

Fig.2 Dynamics of cyst production by a) *Scrippsiella trochoidea*; b) *Biecheleria brevisulcata*; and c) *Levanderina fissa* after treatment with sand particles

0, 0.01, 0.02, and 0.04 g sand particles/well.

3 RESULT

3.1 Effect of sand addition on cyst formation by *S. trochoidea*

The resting cysts of *S. trochoidea* were elliptical, spherical, or oval in shape (15–40 μm diameter), dark brown, with at least one bright red accumulation body and a distinctive double wall with spines on the surface, although spines were absent from some cysts (Fig.1a, b) (Qi et al., 1997; Gu et al., 2008; Wang et al., 2009; Shin et al., 2013).

All wells had the same initial density of vegetative cells at the start of the experiment (764 cells/mL). The production of resting cysts by *S. trochoidea* was significantly higher in the sand treatments (0.02 and 0.04 g) than in the control (Fig.2a; Table 2). Resting cysts were first recorded in sand-treated groups (0.01 g, 0.02 g, and 0.04 g) but not in the control at day 8 of culture. The number of cysts formed in each group increased rapidly over time before reaching a plateau (Fig.2a). Both the final yields and production rates were significantly higher in the sand-treated groups than in the control. The highest mean cyst yield was in the group treated with 0.04 g sand, and was 107-fold that in the control at day 19 of culture (3 093 versus 29 cysts; Fig.2a; Table 2). The cyst production rates and final yields increased as the amount of added sand increased (i.e. 0.04 g> 0.02 g>0.01 g>control; One-way ANOVA, P<0.05, Fig.2a; Table 2). The enhancement effect of sand on cyst production was easily observed by observing the cysts under a microscope (Fig.3a, b).

3.2 Effects of sand on cyst formation by *Biecheleria brevisulcata*

The resting cysts of *B. brevisulcata* K. Takahashi & Iwataki are reported to be dark brown and spherical (diameter, 13.0 to 16.0 μm), with numerous short spines or granule ornamentations on the cell surface (Takahashi et al., 2014). However, the color of cysts was mid-brown rather than dark brown in the

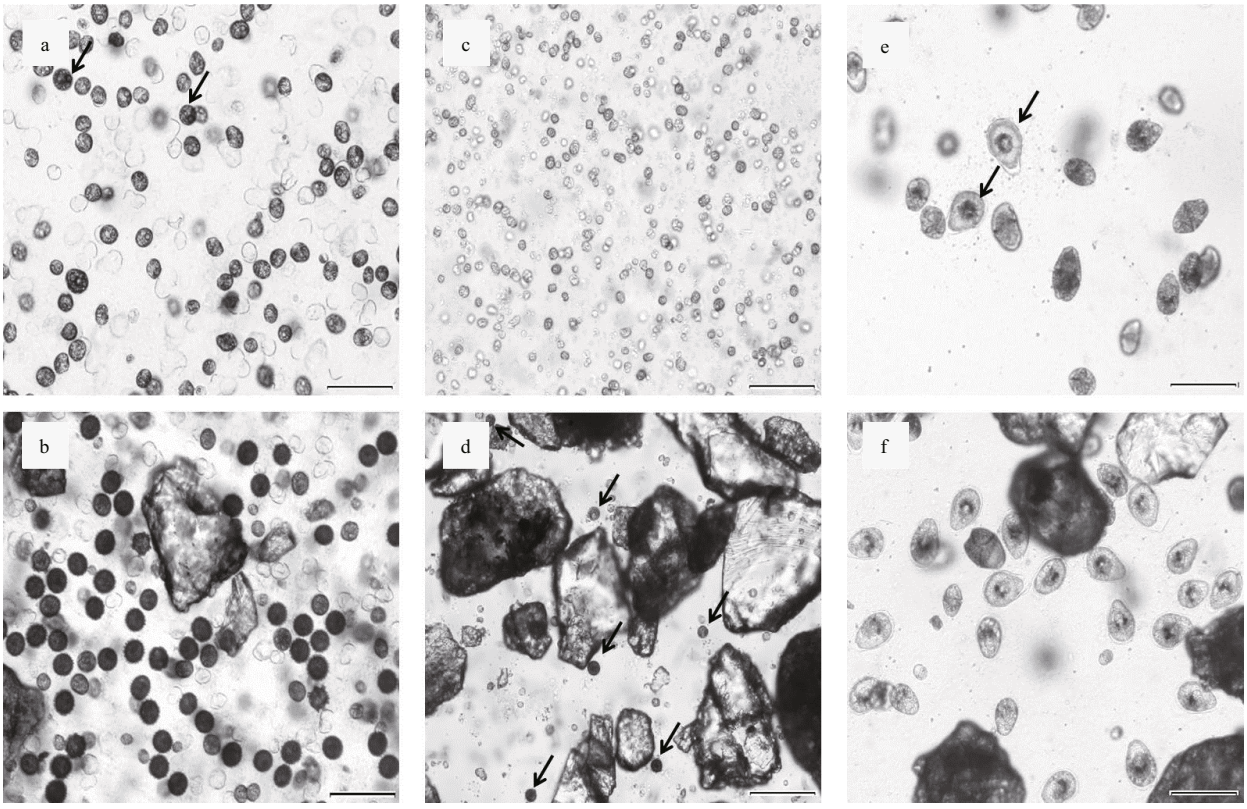

Fig.3 Micrographs of dinoflagellate cysts in controls (a, c, e) and 0.04-g sand treatments (b, d, f), at the end of experiments

a, b. *Scrippsiella trochoidea*; c, d. *Biecheleria brevisulcata*; e, f. *Levanderina fissa* showing difference in cyst density at the bottom of plate wells between control and sand treatments. Arrows indicate cysts (no cysts in Fig.3c, many cysts in Fig.3b, f). All scale bars=100 μm.

laboratory clonal culture in this study (Fig.1c).

All wells contained vegetative cells at the same density at the start of the experiment (2 160 cells/mL). Resting cysts of *B*. *brevisulcata* were first observed on day 6 of culture, and no cysts formed in the control wells (Fig.2b, Fig.3c). The final yields of cysts significantly increased with increasing amounts of added sand (0.04 g>0.02 g>0.01 g>control; one-way ANOVA, $P<0.05$, Fig.2b). In the wells to which 0.01 g and 0.02 g sand was added, the maximum number of resting cysts was on day 12 and 10 of culture, respectively. Some cysts burst for unknown reasons. The highest number of cysts was on day 14 in the 0.04-g sand treatment (533 per well), and this number remained stable until the end of the experiment on day 20 (Fig.2b). The cyst production rate ($R_{avg.}$) varied significantly among the three sand treatments, with the highest production rate (127 cysts per day) in the 0.02-g sand treatment (one-way ANOVA, $P<0.05$, Table 2).

3.3 Effects of sand addition on cyst formation by *Levanderina fissa*

The cysts of *L*. *fissa* (Levander) Moestrup, Hakanen, Hansen, Daugbjerg & Ellegaard are reported to be ovoid (diameter, (54–41) μm×(48–31) μm) and usually flattened on the ventral side, with two thin smooth wall layers (Moestrup et al., 2014). However, we found that the resting cysts had an obvious accumulation body in the central area, and other areas were almost transparent (Fig.1d), as observed by Uchida (Figs.10–13 in Uchida et al., 1996a) and Shikata (Fig.3 in Shikata et al., 2008). This character was not explicitly described by Moestrup et al. (2014, Figs.26–27).

All treatments had the same initial density of vegetative cells (1 146 cells/mL). Resting cysts of *L*. *fissa* formed sooner and faster than did those of the two species above. Resting cysts were observed in all wells from day 1 of culture and reached their maximum levels at day 11 (Fig.2c). The number of cysts in all treatments increased at different rates after day 4, and the final cyst yields were significantly higher in all sand-treated groups than in the control (one-way ANOVA, $P<0.05$). However, the cyst yields did not differ significantly among the three sand treatments (Fig.2c, Fig.3e, f). The mean final cyst yield in the 0.04-g sand treatment was 1.5-fold that in

the control (3 985 vs. 2 715 cysts). However, the cyst production rate did not differ significantly among groups (ANOVA, P=0.065, Table 2).

3.4 Results for other species

The addition of sand particles did not enhance cyst production in three other dinoflagellate species: *Cochlodinium polykrikoides* Margalef, *Akashiwo sanguinea* (Hirasaki) G. Hansen & Moestrup, and *Pheopolykrikos hartmannii* (Zimmermann) Matsuoka et Fukuyo. These experiments were repeated at least twice for each species (data not shown).

4 DISCUSSION

4.1 Addition of sand enhanced production of cysts in some species but not all

Generally, if the clone or species produces cysts easily under normal culture conditions, the addition of sand enhanced cyst formation, as observed in *S. trochoidea*, *B. brevisulcata*, and *L. fissa*. If the culture or species does not produce cysts under normal conditions, or produce few cysts, the addition of sand had only a minimal enhancing effect or no effect. *C. polykrikoides* (Tang and Gobler, 2012), *A. sanguinea* (Tang and Gobler, 2015), and *P. hartmannii* (Matsuoka and Fukuyo, 1986; Tang et al., 2013) have been shown to produce resting cysts in laboratory cultures. The former two species showed relatively low cyst production under the conditions examined, while *P. hartmannii* produced cysts heterothallically (i.e., cysts exclusively produced by clonal crosses but never by individual clonal cultures) (Tang et al., 2013). We conducted pairwise crossing of six clonal cultures of *P. hartmannii*, but none of the crosses produced cysts under these conditions, and the addition of sand did not enhance their cyst production. The resting cysts of *C. polykrikoides* and *A. sanguinea* are generally thin-walled ("pellicles") and therefore, they break easily after formation (Tang and Gobler, 2012, 2015). The addition of sand may have damaged the fragile cysts of these two species.

4.2 Possible mechanisms of enhancement effect

Based on the observations described above, we believe that the enhancement of cyst production was not caused by sand particles altering the genetics or physiology of algal cells. Thus, the addition of sand particles cannot make a cyst non-producer into a producer. The enhancement effect on cyst-producing species depended on that species' inherent potential to produce cysts. One explanation for the mechanism of the enhancement effect is that the sand particles may have increased cell to cell contact. There was limited space in each well, and the dinoflagellate cells moved according to their diurnal migration behavior. The addition of sand particles would have increased the probability of cell collision (cell-cell contact), creating a situation similar to a high cell density environment, but without nutrient competition. The increased rate of cell collision may have triggered the formation of infochemicals that regulate sexual mating or encystment. Although speculative, this explanation is consistent with the observations and explanations of Uchida, who proposed that cell contact may be a possible mechanism underlying cyst formation by dinoflagellates (Uchida, 2001). In addition, Uchida et al. found that more temporary cysts of *Heterocapsa circularisquama* formed when co-cultured with other diatoms (Uchida et al., 1996b) or with *Gymnodinium mikimotoi* at a high cell density (Uchida et al., 1999).

4.3 Possible applications

The methods described in this report can be used to produce large amounts of cysts as experimental materials. For example, transcriptomic studies require a large number of resting cysts to extract sufficient RNA for analyses. More importantly, resting cysts of dinoflagellates can be used to test the efficacy of ships' ballast water treatments. This is a particularly useful test, because dinoflagellates are known to expand their geographical distribution via ships' ballast water (Hallegraeff and Bolch, 1991; Hallegraeff, 1993) and all dinoflagellates that have been transported via ships' ballast water are cyst-producing species (Smayda, 2007). Therefore, when assessing any treatment method of ballast water, it is essential to measure its effects on the viability of resting cysts. Resting cysts generally have a thick wall and relatively low physiological activity, which allow them to resist harsh environmental conditions such as extreme temperatures, prolonged periods of darkness, intense physical pressure, and anoxia. These features make resting cysts a perfect model for testing the efficacy of ballast water treatment technologies. Because these kinds of tests generally involve a large volume of water, a large amount of cysts is required. Therefore, the methods described in this study will be useful to produce a large number of cysts for such analyses.

5 CONCLUSION

Addition of fine sand particles enhanced cyst production in the HAB species *S. trochoidea*, *B. brevisulcata*, and *L. fissa*, but did not induce cyst formation in several other cyst-producing species. Although the underlying mechanisms are unclear at present, the methods used in this study will be useful for producing large amounts of resting cysts for use in research or for large-scale tests. Further research on the mechanisms of the enhancement effect at the molecular level will contribute to our understanding of the processes and regulatory factors of encystment in dinoflagellates.

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