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## Effects of Down- and Up-shocks from Rapid Changes of Salinity on Survival and Growth of Estuarine Phytoplankters

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In the laboratory, we investigated the effects of rapid changes of salinity on the survival and growth of 13 species of phytoplankton inhabiting an estuary where there are wide fluctuations in salinity. These phytoplankton were exposed to rapid decreases in salinity (down-shocks) from 32 to 5, 10, 15, or 20 psu, acclimatized to these lower salinities, and thereafter exposed to rapid increases (up-shocks) from the lower salinities to 32 psu. The phytoplankters tested proliferated in decreases and increases of salinity between 20 and 32 psu in a manner similar to controls (i.e. from 32 to 32 psu), but showed diagnostic responses such as a long lag-time before growth or mortality following drastic decreases or increases between 5–15 and 32 psu. The responses differed among phytoplankton species inhabiting the same estuary. Some flagellates formed morphologically abnormal cells after some treatments involving decreased salinity. The species-specific responses to drastic changes of salinity may contribute to the short-term succession of phytoplankton in estuaries.

### INTRODUCTION

The factors regulating the seasonal succession and bloom events of marine phytoplankters, which are especially harmful algal bloom species, are of great interest both from an ecological perspective and for bloom management. Despite years of monitoring and experimental studies, patterns of succession and bloom events remain largely unpredictable (Anderson and Rengefors, 2006).

Hakozaki Fishing Port, with an average water depth of about 3.0 m, is located in the estuary of the Mikasa River where it flows into Hakata Bay, Fukuoka, Japan (lat 33°37'30"N, long 130°25'00"W). Salinities in this port are usually 32–33 psu, but after heavy rainfall they rapidly decrease to a minimum of 5 psu. Consequently, strong haloclines form; salinity gradients between the surface and bottom layers often exceed 15 psu and sometimes exceed 20 psu. In most cases, within a few days, the halocline rapidly disappeared and the low salinity in the water column recovers to nearly 32 psu. In this fishing port, there are frequent blooms of phytoplankters such as the diatom *Skeletonema costatum*, the raphidophyte *Heterosigma akashiwo*, and the dinoflagellate *Prorocentrum triestinum*.

Many reports have related salinity changes to the

population dynamics of phytoplankton (e.g. Hallegraeff *et al.*, 1995; Weise *et al.*, 2002; Honjo, 2003), and in the laboratory, the effects of salinity on the growth of phytoplankters have been examined both with acclimation to a desired salinity for a certain period of time (e.g. Brand, 1984; Yamaguchi *et al.*, 1997; Nagasoe *et al.*, 2006; Matsubara *et al.*, 2007) and without acclimation (Shimura *et al.*, 1979; Mahoney and McLaughlin, 1979). However, there have been no studies of the combined effects on phytoplankters of down- and up-shocks without acclimation to varying salinity, although it is important to consider the relationships between growth and survival of phytoplankters and salinity in estuaries where the salinity decreases drastically after heavy rain and then increases rapidly. Therefore, to obtain basic information on the short-term succession of phytoplankton species, we investigated the effects of rapid changes of salinity on the survival and growth of 13 phytoplankters that bloom in the Mikasa River estuary.

### MATERIALS AND METHODS

#### Clonal cultures and culture condition

We observed responses to rapid decreases and increases (down- and up-shocks) of salinity in cloned strains of nine diatoms (*Asterionellopsis gracialis*, *Chaetoceros debilis*, *Chaetoceros didymus*, *Chaetoceros socialis*, *Eucampia zodiacus*, *Leptocylindrus danicus*, *Rhizosolenia* sp., *Skeletonema costatum*, and *Thalassiosira minima*), three dinoflagellates (*Akashiwo sanguinea*, *Heterocapsa triquetra*, and *Prorocentrum triestinum*), and one raphidophyte (*Heterosigma akashiwo*). These strains were isolated from an estuary near Hakozaki Fishing Port in Hakata Bay. Subcultures of 13

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of these strains were maintained in modified SWM-3 medium (Yamasaki *et al.*, 2007) at 20 °C and 32 psu under a photon flux density of  $530 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  of cool-white fluorescent illumination on a 12 h:12 h light:dark cycle. The salinities of media used in the experiments were adjusted by diluting aged seawater (34 psu) from the Tsushima Current around Oki Island (lat 34°24'58"N, long 130°12'20"W) with ultra-distilled water. The salinity in the culture medium was measured with a thermosalinity meter (model 85; YSI Inc., Yellow Springs, Ohio, USA). Irradiance in the incubator was measured with a Quantum Scalar Laboratory Irradiance Sensor (QSL-2101; Biospherical Instruments Inc., San Diego, CA, USA).

### Growth experiments

Relative cell abundances in all experiments were measured by *in vivo* fluorescence (unit: fsu) with a Model 10-AU-005-CE fluorometer (Turner Designs, Sunnyvale, CA, USA).

For down-shock experiments, each strain was incubated until cell abundances increased to > 25 fsu at a salinity of 32 psu. Then, 5 mL was inoculated into a 200-mL Erlenmeyer flask containing 95 mL of media adjusted to a salinity of 5, 10, 15, 20, or 32 psu and gently mixed. Four milliliters of culture was then dispensed into each of three polystyrene test tubes (13×10 mm). The cell cultures remaining in the Erlenmeyer flasks and those dispensed into the test tubes were incubated under the same conditions of light and temperature as the original subcultures. Cell abundances in down-shock tests were measured by *in*

*vivo* chlorophyll-*a* fluorescence every day. One milliliter was sampled daily from the cultures in the Erlenmeyer flasks for observation of cell morphology using a light microscope. Photographs were taken of any morphologically abnormal cells observed. Moreover, the cells in the Erlenmeyer flasks were used as a pre-incubation stage for subsequent experiments.

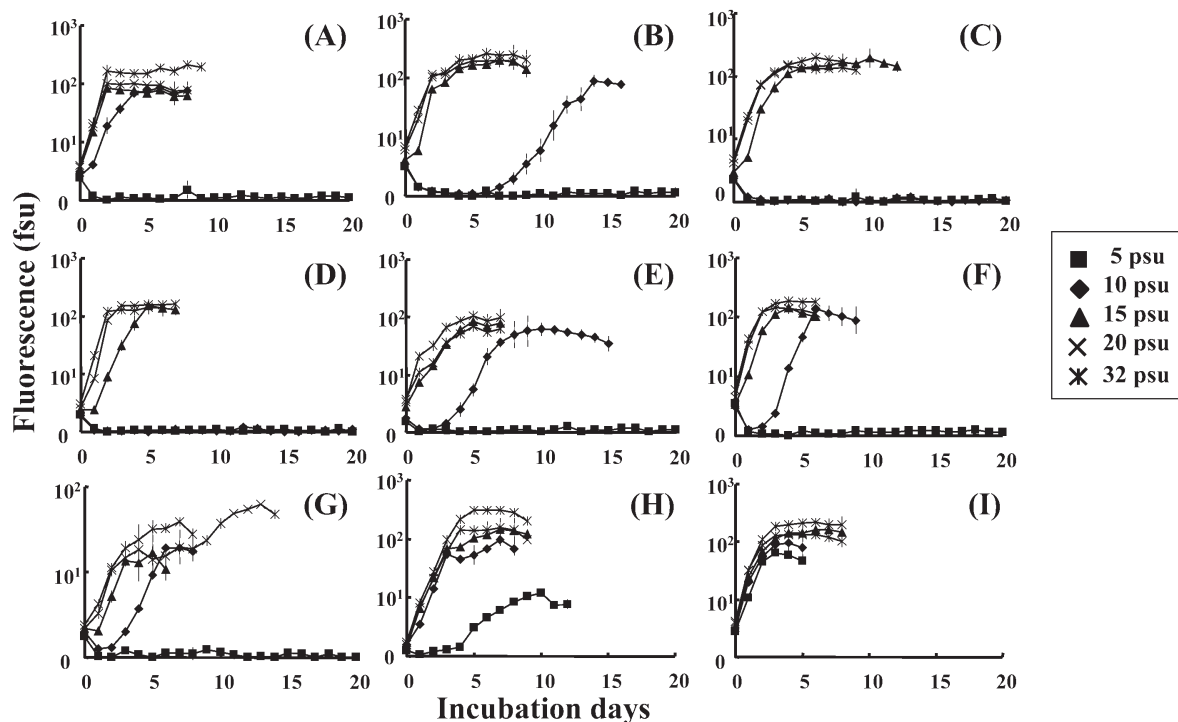
Cells were incubated at reduced salinities in Erlenmeyer flasks until *in vivo* fluorescence exceeded 25 fsu. If the fluorescence of an experimental culture at reduced salinity did not increase to 25 psu, then that culture was not used in the up-shock experiments that followed. If the culture survived incubation at a low salinity, then 5 mL was inoculated into a 200-mL Erlenmeyer flask containing 95 mL of media with a salinity of 32 psu, and gently mixed. Four milliliters of this culture was dispensed into each of three polystyrene test tubes. Thereafter, measurements of cell abundance and observations of cell morphology for the up-shock experiments were conducted daily by using the same methods as in the down-shock tests.

Growth rates ( $\text{div d}^{-1}$ ) during the incubation period were calculated from three consecutive data points by the method of Brand *et al.* (1981), and then the maximum growth rate during the incubation period was determined for each experiment.

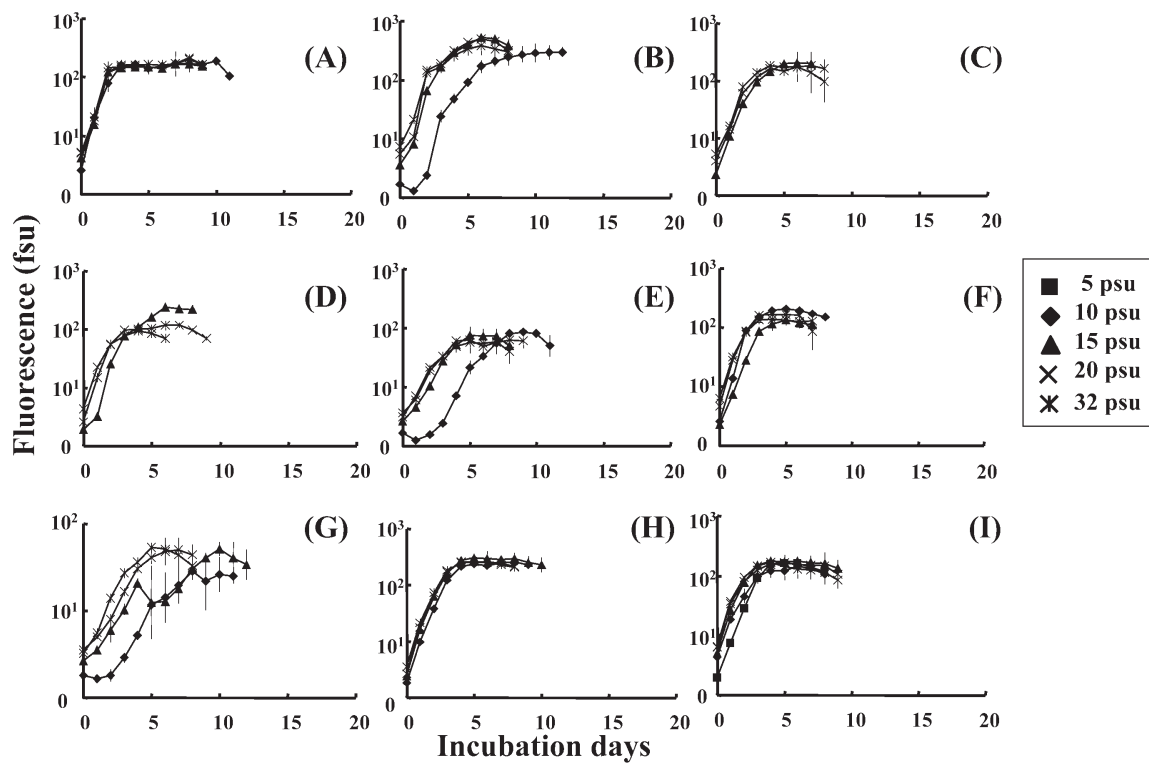
## RESULTS

### Responses of phytoplankters after decreases and increases of salinity

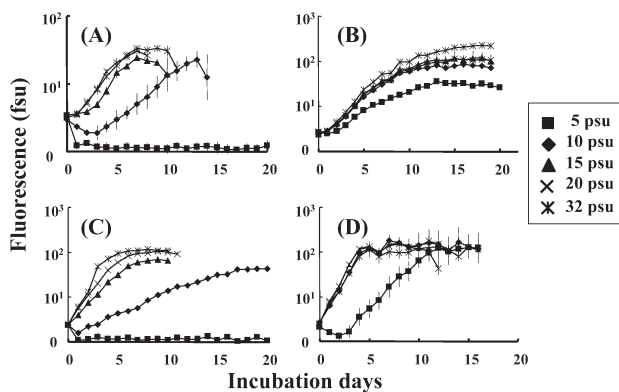
#### Diatoms



**Fig. 1.** Growth curves of diatoms moved from high salinity (32 psu) to low salinities [5, 10, 15, 20, or 32 psu (control)]. (A) *Asterionellopsis gracialis*, (B) *Chaetoceros debilis*, (C) *Chaetoceros didymus*, (D) *Chaetoceros socialis*, (E) *Eucampia zodiacus*, (F) *Leptocylindrus danicus*, (G) *Rhizosolenia* sp., (H) *Skeletonema costatum*, (I) *Thalassiosira minima*.



**Fig. 2.** Growth curves of diatoms moved from low salinities [5, 10, 15, 20, or 32 psu (control)], to which they had acclimated, to a high salinity (32 psu). (A) *Asterionellopsis gracialis*, (B) *Chaetoceros debilis*, (C) *Chaetoceros didymus*, (D) *Chaetoceros socialis*, (E) *Eucampia zodiacus*, (F) *Leptocylindrus danicus*, (G) *Rhizosolenia* sp., (H) *Skeletonema costatum*, (I) *Thalassiosira minima*.



**Fig. 3.** Growth curves of flagellates moved from high salinity (32 psu) to low salinities [5, 10, 15, 20, or 32 psu (control)]. (A) *Akashiwo sanguinea*, (B) *Heterocapsa triquetra*, (C) *Prorocentrum triestinum*, (D) *Heterosigma akashiwo*.

*Skeletonema costatum* and *Thalassiosira minima* proliferated in down-shock experiments at all salinities tested (Fig. 1). However, after the transfer to growth medium at a salinity of 5 psu, the fluorescence of *S. costatum* decreased to below the detection limit (about 0.2–0.3 fsu) and this organism required 4 days before growth was evident. Moreover, the maximum fluorescence of *S. costatum* was low at 5 psu. *Asterionellopsis gracialis*, *Chaetoceros debilis*, *Eucampia zodiacus*, *Leptocylindrus danicus*, and

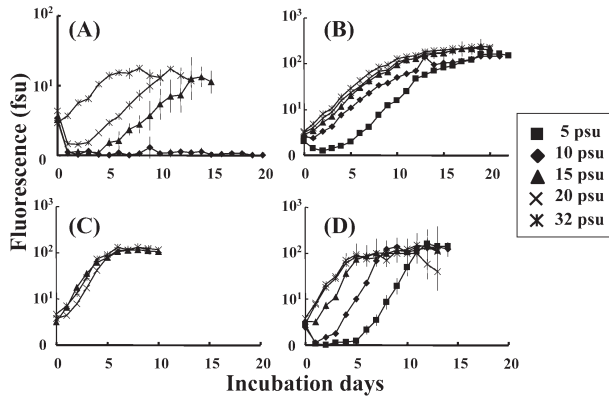
*Rhizosolenia* sp. all proliferated following decreases of salinity from 32 to 10–20 psu, but not to 5 psu. After the decrease to 10 psu, the fluorescence of these species (except for that of *A. gracialis*) decreased to below detection limits and then started to increase within 1–10 days. At the other lower salinities, however, such lag times were not observed. *Chaetoceros didymus* and *C. socialis* proliferated following decreases to salinities  $\geq 15$  psu, but not to 5 and 10 psu; growth was not evident on the first day of incubation at 15 psu.

Because *S. costatum* did not grow at the reduced salinity of 5 psu, an up-shock test involving return to a salinity of 32 psu was not conducted for this species. In all other treatments, diatoms proliferated after being returned to a salinity of 32 psu, much like the control, which was maintained at a salinity of 32 psu throughout (Fig. 2). However, growth of *C. debilis*, *E. zodiacus*, and *Rhizosolenia* sp. was delayed for 1–2 days after the salinity increase from 10 psu to 32 psu, and that of *C. socialis* was delayed for 1–2 days after the increase in salinity from 15 psu to 32 psu.

#### Flagellates

*Heterocapsa triquetra* and *Heterosigma akashiwo* grew at all the salinities used in the down-shock tests (Fig. 3). However, growth at 5 psu was not observed until day 2 or 3. *Akashiwo sanguinea* and *Prorocentrum triestinum* proliferated at 10, 15, 20, and 32 psu, but not at 5 psu. Their growth at 10 psu was first observed on day 3.

*Heterocapsa triquetra*, *P. triestinum*, and *H. akashiwo* all proliferated in all up-shock treatments following their return to 32 psu (Fig. 4). However, *A. sanguinea* did not grow after the increase in salinity

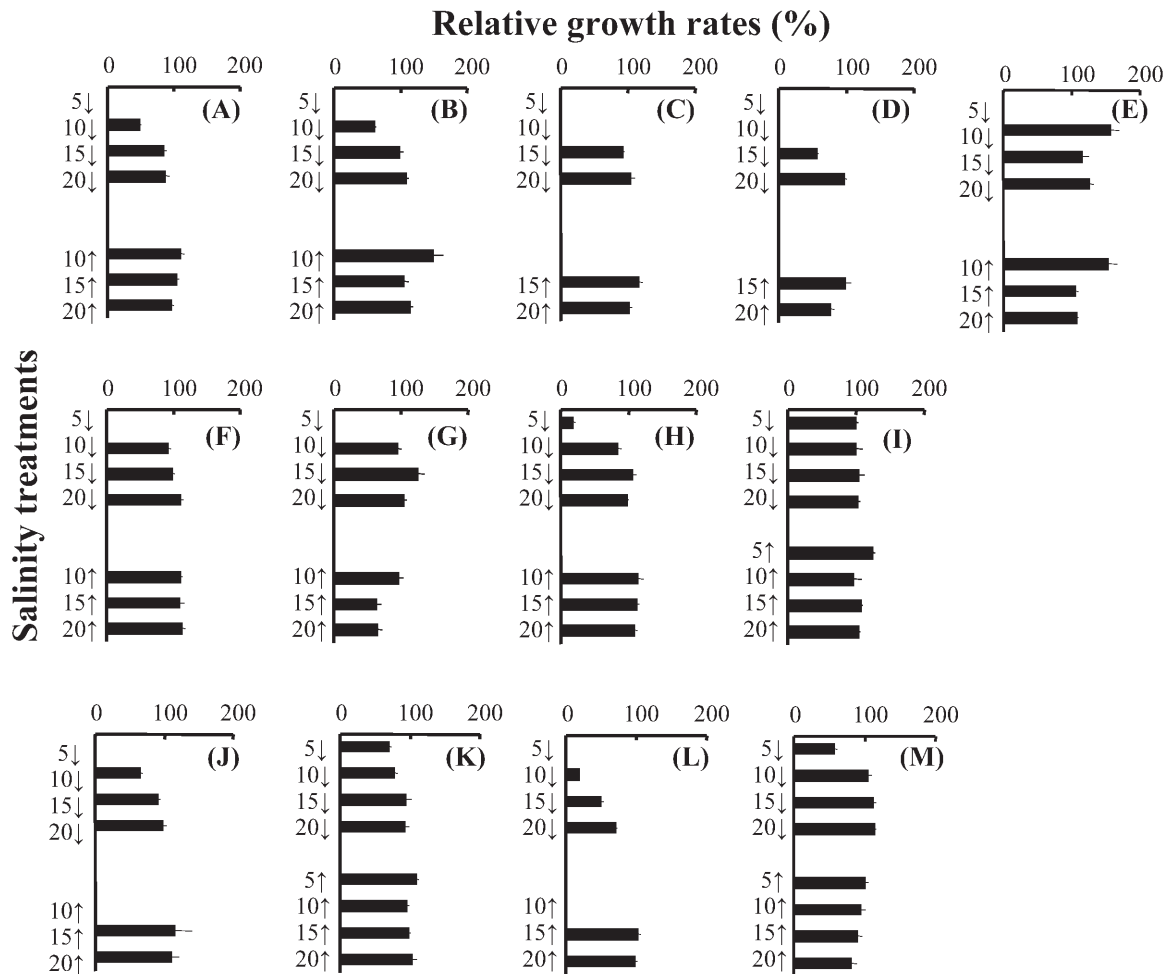


**Fig. 4.** Growth curves of flagellates moved from low salinities [5, 10, 15, 20, or 32 psu (control)], to which they had adapted, to a high salinity (32 psu). (A) *Akashiwo sanguinea*, (B) *Heterocapsa triquetra*, (C) *Prorocentrum triestinum*, (D) *Heterosigma akashiwo*.

from 10 to 32 psu. The fluorescence of *A. sanguinea* and *H. akashiwo* temporarily decreased to below the detection limits following transfers from 15 and 20 psu to 32 psu and from 5 and 10 psu to 32 psu, respectively. *Akashiwo sanguinea*, *H. triquetra*, and *H. akashiwo* had longer lag-times before growth following up-shocks of salinity than those following down-shocks (*A. sanguinea*, 5 days after transfer from 15 to 32 psu and 4 days after transfer from 20 to 32 psu; *H. triquetra*, 5 days after transfer from 5 to 32 psu; *H. akashiwo*, 6 days after transfer from 5 to 32 psu, 3 days after transfer from 15 to 32 psu, and 1 day after transfer from 20 to 32 psu).

#### Growth rates of phytoplankters in exponential phase after decreases and increases of salinity

Growth rates in controls, where salinity was maintained at 32 psu, ranged from 1.22 to 2.75 div d<sup>-1</sup> for the diatoms tested and from 0.53 to 1.41 for the flagellates tested (Table 1). Overall, growth rates of diatoms were higher than those of flagellates. In most cases, the growth rates of the phytoplankters were almost the



**Fig. 5.** Growth rates of phytoplankters tested after different salinity changes. The growth rates are expressed as percentages of controls (i.e. growth rates when salinity was not changed; Table 1). The down arrows represent down-shocks from 32 psu to the salinity indicated. Up arrows are up-shocks, from the salinity listed to 32 psu. (A) *Asterionellopsis gracialis*, (B) *Chaetoceros debilis*, (C) *Chaetoceros didymus*, (D) *Chaetoceros socialis*, (E) *Eucampia zodiacus*, (F) *Leptocylindrus danicus*, (G) *Rhizosolenia* sp., (H) *Skeletonema costatum*, (I) *Thalassiosira minima*, (J) *Akashiwo sanguinea*, (K) *Heterocapsa triquetra*, (L) *Prorocentrum triestinum*, (M) *Heterosigma akashiwo*.



**Table 1.** Growth rates (div d<sup>-1</sup>) of tested phytoplankters at 32 psu

<b>Diatoms</b>	
<i>Asterionellopsis gracialis</i>	2.74
<i>Chaetoceros debilis</i>	2.12
<i>Chaetoceros didymus</i>	2.11
<i>Chaetoceros socialis</i>	2.75
<i>Eucampia zodiacus</i>	1.22
<i>Leptocylindrus danicus</i>	2.17
<i>Rhizosolenia</i> sp.	1.37
<i>Skeletonema costatum</i>	2.50
<i>Thalassiosira minima</i>	2.28
<b>Dinoflagellates</b>	
<i>Akashiwo sanguinea</i>	0.65
<i>Heterocapsa triquetra</i>	0.79
<i>Prorocentrum triestinum</i>	1.41
<b>Raphidophyte</b>	
<i>Heterosigma akashiwo</i>	1.29

same after the decreases and increases of salinity as those of controls without salinity changes (Fig. 5). However, the growth rates of *S. costatum* after a decrease in salinity from 32 to 5 psu, and of *A. gracialis* and *P. triestinum* after salinity decreases from 32 to 10 psu, were less than 50% of those of controls (see Fig. 5).

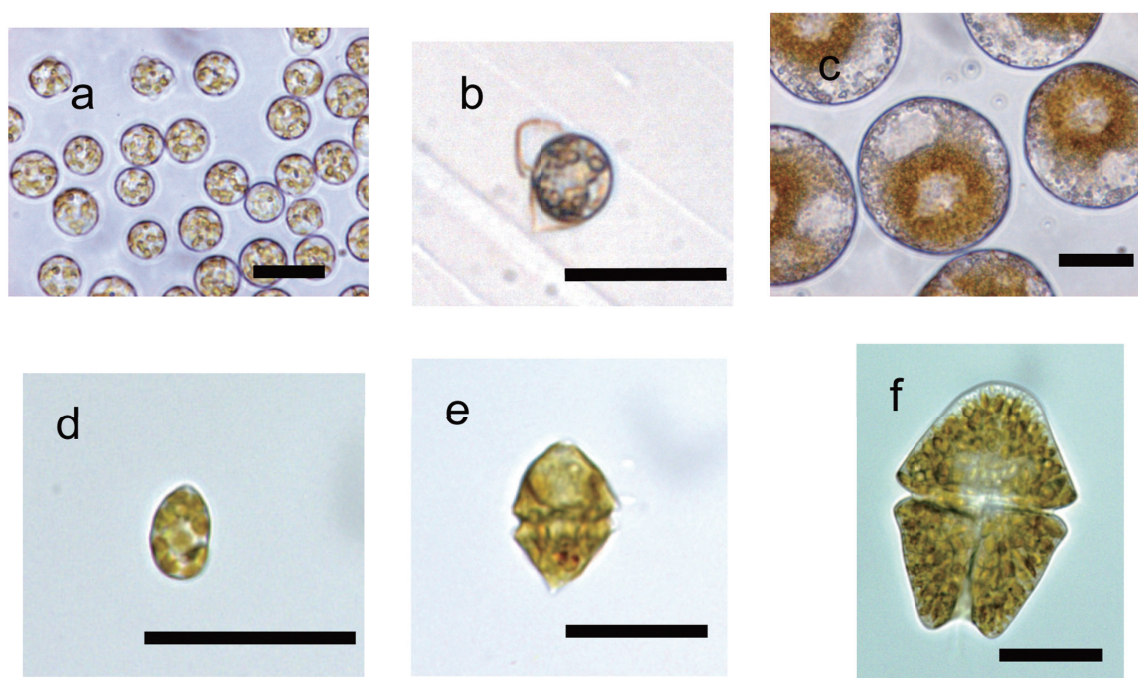
#### Morphological changes of phytoplankton following rapid changes of salinity

For all diatom species and the dinoflagellate *P. triestinum*, all, or a portion, of the cells released their contents and lost pigmentation when they were moved

to salinities at which they could not proliferate either temporarily or through the experimental period. Following decreases and increases of salinity from 32 to 5 psu and from 5 to 32 psu, *A. sanguinea* cells collapsed immediately. All cells of *H. akashiwo* expanded and became spherical (Fig. 6-a), and *H. triquetra* lost its coat and became spherical (Fig. 6-b) as soon as after the decrease of salinity from 32 to 5 psu. Similarly, immediately after the decrease of salinity from 32 to 10 psu, all cells of *A. sanguinea* also expanded and became spherical (Fig. 6-c). Immediately following decreases of salinity, these morphologically abnormal cells of flagellates had almost no motility, but the deformed cells of *H. akashiwo* and *H. triquetra* recovered to normal morphologies by the next day, and those of *A. sanguinea* recovered after an additional day (Figs. 6-d, e and f).

#### DISCUSSION

The growth rates of most of the phytoplankters tested were not substantially different at different salinities (see Fig. 5), a result similar to that from growth experiments involving coastal phytoplankters acclimatized to different salinities (Brand, 1984; Yamaguchi *et al.*, 1997; Nagasoe *et al.*, 2006; Matsubara *et al.*, 2007). Moreover, under rapid decreases and increases of salinity between 20 and 32 psu, all tested phytoplankters proliferated without lag-times, similarly to controls without salinity changes; that is, there were no obvious effects of down- or up-shocks from



**Fig. 6.** Abnormal cells just after salinity was decreased and normal cells when salinity was not changed. (a) *Heterosigma akashiwo* following down-shock from 32 to 5 psu; (b) *Heterocapsa triquetra* following down-shock from 32 to 5 psu; (c) *Akashiwo sanguinea* following down-shock from 32 to 10 psu; (d) *Heterosigma akashiwo* acclimated at 32 psu; (e) *Heterocapsa triquetra* acclimated at 32 psu; and (f) *Akashiwo sanguinea* acclimated at 32 psu. Scale bars=25 μm.

these salinity changes (see Figs. 1–4). Therefore, these phytoplankters, which form blooms in the estuary, have a tolerance to rapid salinity changes between 20 and 32 psu and can easily adapt to them.

However, under the more substantial decreases and increases of salinity between  $\leq 15$  psu and 32 psu, which occasionally occur in Hakozaki Fishing Port, most phytoplankters experienced lag-times before starting to proliferate (see Figs. 1–4). Some decreased in abundance to below the detection limits during these lag-times. For these strains, we suspect that most of the population died just after the rapid salinity decrease or increase, and those few cells that survived recovered and grew after a few days. Marine phytoplankton require time for osmotic regulation following a salinity change (Rijstenbil *et al.*, 1989a, b), and this could also prolong the lag-times.

The abilities to adapt differed among the species tested, as indicated by the salinity ranges that the phytoplankters could tolerate and the lengths of lag-times before growth following rapid salinity changes between  $\leq 15$  psu and 32 psu (see Figs. 1–5). Therefore, considering only salinity changes in the environment, some predictions are possible. If salinity in the estuary rapidly were to decrease from 32 to 10 psu, *C. didymus* and *C. socialis* would completely disappear from the species assemblage, and diatoms such as *A. gracialis*, *S. costatum*, and *T. minima*, and flagellates such as *H. triquetra*, *P. triestinum*, and *H. akashiwo*, all of which have shorter lag-times before growth and higher survival rates than the other phytoplankters tested, would become predominant in the phytoplankton. However, if the salinity were to suddenly decrease from 32 to 5 psu, many species would completely disappear; *S. costatum* might survive but would not recover in large numbers. Nevertheless, under these salinity conditions, *T. minima*, *H. triquetra*, and *H. akashiwo* could survive well and grow at high rates. If the salinity were maintained at low levels, for example at 5 or 10 psu, as caused by a long period of rain, an assemblage composed of *T. minima* and these flagellates might develop, but if the low salinity were to recover immediately to a normal level, *A. sanguinea* and *H. akashiwo*, which do not tolerate salinity up-shocks, would disappear. These presumptions, based on our results, indicate that rapid salinity changes can select those species from a phytoplankton assemblage that will survive and proliferate.

Most flagellates are motile and conduct diurnal vertical migrations (e.g. Hilmer and Bate, 1991; Olsson and Granéli, 1991; Park *et al.*, 2001). The ability to adapt to rapid changes of salinity is required for migration through the halocline. Therefore, some flagellates must have this ability to be able to migrate through the halocline and conduct diurnal vertical migrations (Watanabe *et al.*, 1991; Erga *et al.*, 2003). However, some flagellates decrease their speed of movement when they migrate through strong haloclines (Hilmer and Bate, 1991; Erga *et al.*, 2003).

Following extreme down-shocks of salinity, some

flagellates formed morphologically abnormal cells (see Fig. 6) and almost lost their motility. Although the exact role of the abnormal cells needs further investigation, it is important that we determine the relationship between the adaptations of these phytoplankters to extreme changes of salinity—including any decreases in motile speed, the formation of abnormal cells, and cessation of diurnal vertical migration—if we are to understand the population dynamics of flagellates in estuaries.

In the field, there are many environmental factors that can regulate phytoplankton species succession and biomass (e.g. Karentz and Smayda, 1984; Smayda, 1997; Anderson and Rengefors, 2006). Our results imply different adaptability among species of diatoms and flagellates to rapid and extreme changes of salinity, suggesting that rapid salinity changes could select for a particular species in an existing phytoplankton assemblage; i.e. salinity shock could reset the species composition of the assemblage. Therefore, extreme changes of salinity in estuaries between 32 psu and 5–10 psu could be one of the important environmental factors that determine diatom–flagellate succession and temporal changes in species composition.

#### ACKNOWLEDGEMENTS

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