Growth characterization of non-photosynthetic diatoms, *Nitzschia* spp., inhabiting estuarine mangrove forests of Ishigaki Island, Japan

KEN-ICHIRO ISHI1 & RYOMA KAMIKAWA1,2,*

1 Graduate School of Global Environmental Studies, Kyoto University, Yoshida-nihonmatsu-cho, Kyoto 606–8501, Japan
2 Graduate School of Human and Environmental Studies, Kyoto University, Yoshida-nihonmatsu-cho, Kyoto 606–8501, Japan

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**Abstract:** The non-photosynthetic diatoms *Nitzschia* spp. are known to have evolved from photosynthetic species to heterotrophic species by the loss of photosynthesis. We investigated their ability to tolerate wide ranges of temperatures and salinities. *Nitzschia* spp. were capable of surviving or thriving even at 5°C and 35°C. In addition, these diatoms were also capable of surviving at salinities of 0.5 and 12.0, while thriving at those from 1.0 to 9.0. Such tolerance to a wide range of temperatures and salinities would allow these non-photosynthetic diatoms to thrive in mangrove estuaries, where environmental conditions often drastically fluctuate. Our experiments revealed that the growth rates of the non-photosynthetic diatoms were larger than those estimated based on cell volumes and temperature, suggesting that these non-photosynthetic diatoms may be an important group of organisms contributing to material circulation by growing heterotrophically in mangrove estuaries.

**Key words:** growth rates, heterotrophs, *Nitzschia*, mangroves, temperature, salinity

**Introduction**

Through photosynthesis, primary production by algae in the ocean generates large amounts of molecular oxygen, indispensable for aerobic organisms on Earth. The carbon fixed through this primary production supports the food web. Not only planktonic species, but also benthic microalgal communities present in tidal areas also contribute significantly to primary production (e.g. MacIntyre et al. 1996, Meyercordt & Meyer-Reil 1999, Ichimi et al. 2008, Scholz & Liebezeit 2012). Both ecological and physiological characterizations suggest that benthic microalgae are a major food source for phagotrophic species in the ocean (Round 1971, Admiraal 1984, McIntire & Moore 1977, Miller et al. 1996, Underwood & Kromkamp 1999).

In tidal areas like mangrove estuaries, physical environmental conditions such as temperature and salinity drastically changes over relatively short terms (e.g., Karsten et al. 1996, Scholz & Liebezeit 2012). This rapid environmental fluctuation exposes the benthic microalgae to unfavorable environments. For example, microalgae thriving in tidal areas will be exposed directly to high light intensity when the seawater has receded and surfaces dry. This evaporation phenomenon also triggers other severe conditions such as high temperature and high salinity. Nevertheless, it has been reported that various algal species are capable of inhabiting such severe environments (Williams 1964, Mizuno 1992, Colijn & van Buurt 1975, Admiraal & Peletier 1980, Pinckney & Zingmark 1991, Kamikawa et al. 2015a).

Bacillariophyceae (diatoms) is a group of microalgae that contribute approximately 20% of annual primary production worldwide (Field et al. 1998, Mann 1999). Regardless of the benefits of photosynthesis for autotrophic life through carbon fixation, some diatom species have lost the ability and have become heterotrophic osmotrophs. The diatom genus *Nitzschia* is benthic and is comprised of more than 1,000 species, containing 6 non-photosynthetic species (Guiry & Guiry 2017, Lewin & Volcani 1987, Li & Volcani 1987). Our previous phylogenetic analyses suggested that the loss of photosynthesis in *Nitzschia* has happened multiple times independently (Kamikawa et al. 2015a). Transmission electron microscopy revealed that
some of these species have retained non-photosynthetic plastid structures with reduced thylakoids. We found non-photosynthetic diatoms have plastid genomes that are streamlined by losing genes and lack any genes for photosystems I and II, cytochrome $b_{6}/f$ complex, and carbon fixation (Kamikawa et al. 2015b).

We have investigated the diversity of non-photosynthetic diatoms (Kamikawa et al. 2015a). We isolated and established non-photosynthetic diatom strains from the tidal estuarine areas of mangroves in the Ryukyu Islands, Japan. Although non-photosynthetic diatoms are undoubtedly involved in material circulation as consumers not as primary producers (Blackburn et al. 2009a, b), we still have no data to explain how and why these non-photosynthetic Nitzschia spp. can survive and even thrive in the tidal areas of mangrove forests, where the environmental conditions drastically fluctuate. In previous studies, the growth rates and salinity tolerances of non-photosynthetic diatoms Nitzschia spp. isolated from the mangroves and an intertidal sandy beach in Florida were investigated and they were shown to grow rapidly and to be tolerant to salinities of 1.0–6.0 (Blackburn et al. 2009a, b). To the best of our knowledge, however, there have been no reports on the growth characteristics of non-photosynthetic diatoms isolated from mangrove forests in the Ryukyu Islands, Japan. In this study, we report high growth rate of these non-photosynthetic diatoms and their tolerances to wide ranges of temperatures and salinities. The above might explain why these non-photosynthetic diatoms are capable of growing under such rapidly fluctuating environmental conditions. We propose that the non-photosynthetic Nitzschia spp. are highly adapted to the tidal estuarine areas of mangrove forests in the Ryukyu Islands, and that they are possibly one of the major consumers contributing to material circulation there.

Materials and Methods

Collection, establishment, and maintenance of cultures

Sand and mangrove leaves were collected from the Nagura-ampal estuary (24°40′24.8″N; 124°14′17.1″E) and the Miyara-river estuary (24°35′38.7″N; 124°21′11.7″E) of Ishigaki Island in Okinawa, Japan (Fig. 1), on 18th February 2016. Each of these samples was inoculated in autoclaved seawater and maintained at 20°C in dark conditions. Single cells of colorless diatoms were isolated by micro-pipetting and were cultured in the Daigo IMK medium (Nihon Pharmaceutical Co., Tokyo, Japan) made with artificial seawater (Marine Art SF-1; Tomita Pharmaceutical Co., Tokushima, Japan) for one week in a 12-well Tissue Culturing Plate (1 ml for each well; BD Falcon, Tokyo, Japan). Fifty micrograms each of kanamycin and ampicillin were added into the 1 L medium to restrict any contaminating bacterial growth. After incubation for a week, sterility was checked using direct observation with DAPI (4′,6-diamidino-2-phenylindole) staining and epifluorescence microscopy (Porter & Feig 1980, Imai 1984). If bacterial cells were observed, a diatom cell was isolated from the well and the above procedures for sterilization were repeated. Established strains were maintained in the Daigo IMK medium under dark conditions at 20°C. The four strains used in this study are summarized in Table 1. It is worth noting that the IMK medium is a medium originally developed for autotrophic microorganisms and it contains only ethylenediaminetetraacetic acid and vitamins as, if available, candidates for carbon sources. In a preliminary experiment, we were unable to observe any differences in

Table 1. Strains used in this study. Lineages were identified with morphology and LSU rRNA gene sequences according to Kamikawa et al. (2015a). Growth rates estimated at 30°C during the logarithmic growth phase within 48 h after inoculation are shown. Growth rates only at 30°C are shown here, since rates at the other temperatures were lower in every strain.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Lineages*</th>
<th>Origins</th>
<th>Locations</th>
<th>Sampling date</th>
<th>Growth rates $[\mu_{\text{max}} \text{ (day}^{-1})]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL1-1</td>
<td>1</td>
<td>A mangrove leaf on river bed</td>
<td>Nagura-ampal estuary</td>
<td>Feb 18th, 2016</td>
<td>3.0</td>
</tr>
<tr>
<td>PL1-4</td>
<td>4</td>
<td>A mangrove leaf on tidal flat</td>
<td>Miyaragawa estuary</td>
<td>Feb 18th, 2016</td>
<td>2.5</td>
</tr>
<tr>
<td>PL2-3</td>
<td>6</td>
<td>A mangrove leaf on tidal flat</td>
<td>Miyaragawa estuary</td>
<td>Feb 18th, 2016</td>
<td>2.9</td>
</tr>
<tr>
<td>PL3-3</td>
<td>3</td>
<td>A mangrove leaf on tidal flat</td>
<td>Miyaragawa estuary</td>
<td>Feb 18th, 2016</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*: Corresponding to Lineages described in Kamikawa et al. (2015a).
growth rates of the non-photosynthetic *Nitzschia* sp. NIES-3581 in the IMK medium and the IMK medium containing 0.001–10 g L$^{-1}$ of glucose (data not shown). We used the IMK medium for the following experiments to investigate growth potential of the non-photosynthetic *Nitzschia* spp.

**Identification of established strains**

Identification was performed by morphological observation under light microscopy according to the criteria reported in Kamikawa et al. (2015a). All strains were observed using an inverted microscope (Olympus IX70, Tokyo, Japan) equipped with a FLOYD multi interface digital camera (WRAYMER, Osaka, Japan). We sequenced the nuclear LSU rRNA genes as reported in Kamikawa et al. (2015a) using the primer set (D1R-F: 5′-ACC CGC TGA ATT TAA GCA TA-3′; Scholin et al. 1994, D3B-R: 5′-CCT TGG TCC GTG TTT CAA GA-3′; Nunn et al. 1996) and the polymerase chain reaction (PCR) conditions described in Lundholm et al. (2002). Nucleotide sequences were subjected to a homology-based search by blastN in the GenBank database (https://www.ncbi.nlm.nih.gov/) and confirmed to be completely identical to the nuclear LSU rRNA gene sequences from non-photosynthetic diatom lineages (Kamikawa et al. 2015a).

**Measurement of instantaneous growth rate**

To determine growth characteristics at different water temperatures, a cultivation experiment was carried out in 1 mL Daigo IMK medium with a salinity of 3.0 at 5, 10, 15, 20, 25, 30, 35 and 40°C in dark conditions. Ten microliters of each strain culture in log phase growth at 20°C under a salinity of 3.0 was, without acclimation to each temperature condition, inoculated into 990 µL of the fresh medium in a well for each experiment. Cells attached to the surface of each well were counted in triplicate by observation using an inverted microscope every day for a week. The resultant cell numbers were converted into cell density (cells L$^{-1}$). The growth rate was estimated from the following equation: $(\ln N_2-\ln N_1) t^{-1}$, where $N_2$ and $N_1$ are the averaged cell densities (cells L$^{-1}$) at 24 and 0 h, respectively, and $t$=time (days), according to Stanier et al. (1976). The growth rate was converted to generation time (days) using the following equation: $\ln(2) (\ln N_2-\ln N_1)^{-1}$ t (Stanier et al. 1976). Therefore, a division rate (day$^{-1}$) was calculated by using the following equation: $(\ln N_2-\ln N_1) [\ln(2)]^{-1} t^{-1}$. If explicit growth in a strain culture was not observed, the culture was incubated for another one week at a salinity of 3.0 in dark conditions, in order to confirm whether cells were alive.

To determine growth characteristics under different salinity conditions, cultivation experiments were carried out at salinities of 0.5, 1.0, 3.0, 6.0, 9.0, 12.0, 15.0, 18.0 and 21.0 at 20°C in dark conditions. Salinity concentrations were adjusted by dilution with distilled water by adding the saturated salt water (i.e. 26.4% NaCl) at 20°C. Ten microliters of each strain culture under log phase growth was, without acclimation to each salinity condition, inoculated into fresh 1 mL Daigo IMK media that included 1 mM Na$_2$SiO$_3$. Cell density (cells L$^{-1}$) was measured as described above. If explicit growth in a strain culture was not observed, cells were incubated for another one week at a salinity of 3.0 in dark conditions, in order to confirm whether cells were alive.

**Results**

**Identification of newly established culture strains**

The previous study reported that non-photosynthetic diatoms found in the mangroves around the Ryukyu Islands could be classified into lineages 1–6, on the basis of morphology and nuclear LSU rRNA genes (Kamikawa et al. 2015a). Light microscopic observations divided our newly established culture strains into 4 types (Fig. 2). Strain PL1-1 was classified as lineage 1, PL1-4 was lineage 4, PL2-3 was lineage 6, and PL3-3 was lineage 3, according to Kamikawa et al. (2015a). Consistent with the morphology, nuclear LSU rRNA gene sequences of PL1-1, PL1-4, PL2-3, and PL3-3 were completely identical to those of lineages 1, 4, 6, and 3, respectively (Table 1). All diatoms used in this study were isolated only from mangrove leaves on the river bed or tidal flat.

![Fig. 2. Microphotographs of the 4 types of benthic diatoms used in this study. a. girdle face of a cell of strain PL1-1. b. valve face of a cell of strain PL1-4. c. girdle face of a cell of strain PL2-3. d. girdle face of a cell of strain PL3-3.](image-url)
Growth potential of non-photosynthetic diatoms

Temperature range

In order to evaluate the tolerance of the four non-photosynthetic diatom strains to low or high temperatures, we cultivated these strains under eight different temperature conditions. The mean cell densities at the different water temperatures are shown in Fig. 3. We observed cell growth of PL1-1, PL1-4, PL2-3, and PL3-3 in the temperature range from 15°C to 35°C. All the strains showed the highest growth rates (day \(^{-1}\)) at 30°C (Fig. 3; Table 1). At 30°C, the growth rate of the PL1-1 strain was 3.0 day \(^{-1}\). The growth rate of the PL1-4 strain was 2.5 day \(^{-1}\), which was slightly lower than that of the other strains. PL2-3 grew at a rate of 2.9 day \(^{-1}\). In strain PL3-3 it was 4.2 day \(^{-1}\), which was the highest growth rate of the strains investigated.

At 5 and 10°C, none of the strains grew explicitly (Fig. 3). In order to determine whether or not the cells were capable of surviving at those lower temperatures, we then incubated those cultures at 20°C under dark conditions for one more week in addition to the one-week cultivation at the lower temperatures. As a result, those cultures began to grow again at 20°C (Table 2), indicating that the non-photosynthetic diatoms had been able to survive at 5 and 10°C for one week, at least under those laboratory conditions. Similar experiments were performed for the cultures at 40°C. We did not observe any growth after incubation at 20°C for a week following the one-week cultivation at 40°C. Thus, all the strains were incapable of growing, or even surviving, at 40°C, as shown in Fig 3 and Table 2. Through these experiments, we conclude that PL1-1, PL1-4, PL2-3, and PL3-3 are capable of surviving from 5 to 35°C.


Table 2. Growth and survival at different water temperatures and salinities. Details of experiments at water temperatures between 15–35°C are shown in Fig. 3.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Water temperature (°C)</th>
<th>Salinity</th>
<th></th>
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<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>15–35</td>
<td>40</td>
<td>0.0</td>
<td>0.5</td>
<td>1.0</td>
<td>3.0</td>
<td>6.0</td>
<td>9.0</td>
<td>12.0</td>
<td>15.0 ≤†</td>
</tr>
<tr>
<td>PL1-1</td>
<td>*</td>
<td>*</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>PL1-4</td>
<td>*</td>
<td>*</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>PL2-3</td>
<td>*</td>
<td>*</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>PL3-3</td>
<td>*</td>
<td>*</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>*</td>
</tr>
</tbody>
</table>

+: thriving, *: not thriving but surviving, −: dead, †: 18.0 and 21.0 also examined.
Salinity range

In order to evaluate the tolerance to salinity, we performed growth experiments under different salinity conditions. Growth of the non-photosynthetic diatoms was observed at salinities from 1.0 to 9.0 in all the strains used (Table 2). In contrast, none of the strains grew at salinities of 12.0 or higher. However, cell growth at a salinity of 0.5 varied among the strains (Table 2). PL1-4 and PL2-3 grew at a salinity of 0.5, whereas PL1-1 and PL3-3 did not show any growth at this salinity (Table 2). In order to determine whether or not these non-photosynthetic diatom strains were capable of surviving at salinities ≤0.5 or ≥12.0, we incubated the cultures at a salinity of 3.0 under dark conditions for one additional week. As a result, we observed growth of PL2-3 and PL3-3, which had been cultivated at a salinity of 12.0, in this additional experiment. However, PL1-1 and PL1-4 cultivated at a salinity of 12.0, PL1-1 and PL3-3 cultivated at a salinity of 0.5, and all strains cultivated at salinities of 0 and ≥15 did not grow (Table 2), indicating that they are incapable of surviving under those salinity conditions. These results show that PL1-1, PL1-4, PL2-3, and PL3-3 are capable of growing under salinities of 1.0–9.0, while the lowest and highest salinity concentrations at which they can survive vary amongst the strains.

Discussion

Temperature tolerance

Diatoms are known to have adapted to a wide range of temperatures (e.g., Thomas 1966, Colijn & van Buurt 1975, Admiraal & Peletier 1980, Suzuki & Takahashi 1995, Mock & Hoch 2004, Boyd et al. 2013, Thorel et al. 2014, Woelfel et al. 2014). The non-photosynthetic diatoms investigated in the present study are capable of surviving at temperatures from 5 to 35°C, which is wider than that reported for a strain of a closely related species, such as *Pseudo-nitzschia australis* Frenquelli, which has been reported to be capable of growing from 5 to 20°C (Thorel et al. 2014). Therefore, the temperature range to which these non-photosynthetic diatoms are tolerant allows them to be regarded as eurythermal. It is known that temperatures around Ishigaki Island, located in the subtropical zone, occasionally decrease to less than 10°C, as in an exceptional case that was observed in January 2016 by the Japan Meteorological Agency (http://www.data.jma.go.jp/obd/stats/etrn/view/annually_s.php?prec_no=91&block_no=47918&year=&month=&day=&view=). Accordingly, it seems beneficial for species inhabiting mangrove estuaries, where local evaporation would result in higher salinity conditions. Comparable tolerance to higher salinities has also been reported for the diatoms *Amphora cf. subacutuscula* Schoeman, *Nitzschia fusiformis* Gruwow, and *Entomoneis* sp., isolated from thalassic hypersaline marine environments (Clavero et al. 2000). Given that these variable species are phylogenetically only distantly related to each other, adaption to high salinity conditions could have arisen in various diatom lineages independently. Many other diatoms have been shown to be incapable of thriving or even surviving at a salinity of 9.0 (Williams 1964, Admiraal 1977, Underwood & Provot 2000, Woelfel et al. 2014). It is worth noting that two benthic diatom species *Nitzschia sigma* (Kützing) W. Smith and *Navicula arenaria* Dokin have been confirmed to grow well in salinities ranging from 0.2 to 4.5, and 0.8 to 4.5, respectively (Admiraal 1977). Even compared to *N. sigma* and *N. arenaria* closely related to the non-photosynthetic *Nitzschia* spp., the non-photosynthetic diatom strains investigated here can be characterized as euryhaline organisms.

High division rates

The non-photosynthetic diatoms investigated in this study showed maximum growth rates between 2.5 and 4.2 day⁻¹ at 30°C. Comparably high growth rates were estimated from non-photosynthetic diatoms isolated from mangroves in Florida (Blackburn et al. 2009a), support-
ing the idea that high growth rates are common in non-photosynthetic diatoms. The measured growth rates of 2.5 and 4.2 day$^{-1}$ imply the diatoms strains used in this study have the potential to divide 3.6–6.1 times in a day. Although growth rates have been measured for many photosynthetic benthic diatom strains isolated from various regions, such as Nitzschia sigma (Kützing) W. Smith and Navicula arenaria Donkin from estuaries (Admiraal 1977) and Navicula perminuta Grunow, Melosira moniliformis (O.F. Müller) Agardh and Nanofrustulum shiloi Lee, Reimer & McEmery from brackish sandy sediments (Woelfel et al. 2014), their growth rates were much lower than those of the present non-photosynthetic diatoms. For example, divisions day$^{-1}$ of benthic diatoms isolated from the brackish southern Baltic Sea were determined to be 0.3–1.5 at 30°C (Woelfel et al. 2014).

Although higher growth rates ($\mu_{max}$) have been reported in other algae such as the diatoms Chaetoceros gracilis Schütt (4.2 day$^{-1}$; Thomas 1966) and Chaetoceros sal-sugineum Takano (8.4 day$^{-1}$; Ichimi et al. 2012) in which cell diameters are 3–6 $\mu$m, the high growth rates of the non-photosynthetic diatoms are remarkable when their cell sizes are considered. Montagnes & Franklin (2001) proposed an equation explaining the relationship between the growth rate of diatoms, temperature, and cell volume

$$\log_{10} V = -0.544 + 0.0206 \times T - 0.0864 \times \log V \left( \mu m^3 \right).$$

The cell volume of Nitzschia sp. lineage 1 (PL1-1), with a lanceolate frustule with an apical axis of 16.5 $\mu$m, a transapical axis of 2.0 $\mu$m, and a pervalvar axis of 1.0 $\mu$m, is estimated to be ca. 16.5 $\mu m^3$, according to the equation proposed by Hillebrand et al. (1999). Thus, the growth rate of PL1-1 is expected to be ca. 1.1 day$^{-1}$ at 30°C according to Montagnes & Franklin (2001). However, the experimentally determined growth rate of PL1-1 is 3.0 day$^{-1}$ (Table 1), which is a much higher rate than the ca. 1.1 day$^{-1}$ estimated by the equation. The estimated (calculated) growth rates of the other strains, namely Nitzschia spp. PL1-4, 2-3 and 3-3, are expected to be much smaller than PL1-1, given their larger cell volumes (Fig. 2). However, the experimentally confirmed growth rates of strains PL1-4, 2-3 and 3-3 are higher than or only slightly lower than that of PL1-1 (Table 1). These inconsistencies between estimated growth rates and the experimentally confirmed rates draw attention to the unexpectedly high growth rates of these non-photosynthetic diatoms, given their cell volumes.

Environmental conditions in the mangrove estuaries of Ishigaki Island fluctuate drastically (e.g., Karsten et al. 1996). Since no acclimation to the temperatures and salinities used in this study was conducted before inoculation, the non-photosynthetic diatoms seemed tolerant not only to wide ranges of temperatures and salinities but also to rapid environmental fluctuations. The non-photosynthetic diatoms investigated in this study were found to be both eurythermal and euryhaline, likely because of their evolutionary adaptation to the tidal estuaries where mangroves occur. In order to determine precisely to what degree the environmental conditions fluctuate in microhabitats populated by non-photosynthetic diatoms, more work needs to be carried out.

Given their high growth rates and relatively large cell sizes, it is very likely that they contribute, to some degree, to material circulation in mangrove areas. As non-photosynthetic Nitzschia spp. have been reported from both Ishigaki Island and Iriomote Island (Kamikawa et al. 2015a), these heterotrophic diatoms might contribute ecologically to multiple mangrove-inhabited areas in the Ryukyu Islands. Therefore, it is important to investigate more accurately the distribution and seasonal abundance of such non-photosynthetic diatoms through field work in the future, in order to know how much they contribute to material circulation in these mangrove estuaries.

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**References**


Guiry MD, Guiry GM (2017) Algae Base. World-wide electron-
ic publication, National University of Ireland, Galway. http://www.algaebase.org (searched on 22 February 2017)


