**Variation in the abundance of periphytic algae in marine biofilms on glass surfaces submerged in the sea off Shin-Nagasaki Port, Nagasaki, Japan**

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(Received May 6, 2016; Accepted July 29, 2016; Published Online November 10, 2016)

**Abstract**

Glass slips were submerged in the sea (1 m depth) off Taira-cho, Nagasaki, Japan for 1, 2, 3 and 4 weeks every month from Jul 2010 to Oct 2012, and the dry weights, chlorophyll contents and diatom community structures of the marine biofilms were investigated. Glass slips immersed in the sea acquired biofilms that consisted mainly of diatoms. Young thalli of *Ulva compressa* also occurred in the biofilms from Apr to Dec. Marine biofilms increased in mass with longer immersion periods, indicating that growth of marine biofilms should be looked into for up to a month. Colonization on glass slips by invertebrate macroorganisms occurred from Jul to Sep, consequently making the estimation of biofilm biomass inaccurate during this period. Dry weights of biofilms were linearly correlated with the amounts of chlorophyll-α and -c, indicating that these pigments can be used as indices to estimate biomass growth of marine biofilms, which primarily consist of periphytic microalgae. Chlorophyll-α contents and diatom densities of biofilms both showed temporal and seasonal variations, whereas chlorophyll-c contents of biofilms showed seasonal variation. Decrease in the chlorophyll (α and -c) contents and diatom densities of biofilms in Jul to Sep may probably be due to the disturbance of biofilms caused by the attachment of invertebrate macroorganisms. *Navicula* almost always was the dominant diatom in established marine biofilms.

**Keywords**: marine biofilm, biofilm growth, periphytic diatoms, chlorophyll-α, chlorophyll-c, algal biomass

**Introduction**

Surfaces submerged in the sea initially acquire a thin layer of organic materials, referred to as a conditioning film, which is then colonized by bacteria, fungi, diatoms and protozoans, the main organisms that comprise marine biofilms (Wahl, 1989). Colonization by these micro-organisms follows no particular order (Marszalek et al., 1979).

In benthic ecosystems, marine biofilms are known to be an important source of chemical cues that either facilitate or inhibit settlement of algal spores (e.g. Joint et al., 2000; Silva-Aciares and Riquelme, 2008; Shin, 2008) and invertebrate larvae (see review by Qian et al., 2007; Dobretsov et al., 2013). While bacteria influence larval settlement by their production of waterborne-metabolites (e.g. Fitt et al., 1989; 1990; Bao et al., 2007a), extracellular polymeric substances (EPS) produced by diatoms in biofilms have been shown to induce larval settlement in some studies (e.g. Lam et al., 2003; 2005; Jouuchi et al., 2007). Jouuchi et al. (2007) reported that an LCA-binding sugar chain compound produced by an epiphytic diatom *Navicula ramosissima* induced larval settlement in the barnacle *Amphibalanus amphitrite*. Biofilms are also utilized by grazers as food. Chapman (1981) reported that where kelp is absent, sea urchins in St. Margaret’s Bay, Eastern Canada fed on benthic diatoms.

From an economic standpoint, biofilms are considered a nuisance to maritime industries because it is a precursor phenomenon to the biofouling of macro-organisms and it influences corrosion of metallic materials leading to economic losses (Yebra et al., 2004). On the other hand, hatcheries in Japan typically grow diatom-based biofilms on artificial substrates to induce larval settlement and feed juveniles of sea urchins (Ito, 1984), sea cucumbers (Ito and Kitamura, 1997) and abalones (Kawamura et al., 1995; Takami et al., 1996; 1997). Success in the production of these economically valuable species will rely on how hatcheries can control production of diatom-based biofilms that will consistently yield high settlement of larvae and good quality food for juveniles. In this regard, understanding the process of biofilm development on submerged surfaces will ultimately find application in controlling biofilm growth to benefit aquaculture and other maritime industries.

In the marine environment, diatoms will grow and become the major component of the biofilm where light is sufficient (Caron and Sieburth, 1981). The abundance of benthic microalgae and diatom assemblages have been shown to be influenced by surface (Marszalek et al., 1979; Patil and Anil, 2005), season (Patil and Anil, 2005), time (Nayar et al., 2005; Patil and Anil, 2005; Satheesh and Wesley, 2012), and plank-
tonic blooms (Lukatelich and McComb, 1986; Patil and Anil, 2005). These studies investigated the process of biofilm development during a period of 5 to 14 days by following diatom growth and changes in their community structures for 1 year. Campbell et al. (2011) suggested that studying biofilm growth for longer time periods (weeks instead of days) would provide a more realistic timeframe for biofilm development; and this would be more representative of the natural processes that occur on submerged surfaces. In this regard, we investigated the changes in the total dry weight, chlorophyll contents and the diatom community assemblage of marine biofilms during their development on glass surfaces submerged in the sea for 1 to 4 weeks from July 2010 to October 2012. The purpose was not only to collect additional information on the variation in abundance of periphytic microalgae, particularly diatoms during biofilm development, but also to investigate the validity of using chlorophyll contents as a biomass index for measuring marine biofilm growth. The investigation was carried out for a period of more than 2 years in order to obtain reliable results on the effects of seasons on biofilm development.

Materials and Methods
Study site and preparation of marine biofilms

Glass slips used in the investigation were glass microscope slides that were each cut to a 38 mm × 26 mm dimension. These were washed, first with tap water and then with distilled water (DW), air-dried and set on PVC holders. These PVC holders were then submerged in the sea from a raft adjacent to the Nagasaki Prefectural Institute of Fisheries, Tairamachi, Nagasaki, Japan (129°46' E, 32°48' N, Fig. 1). PVC holders with the glass slips were immersed at a depth of about 1.0 m below the sea surface every week from July 2010 to October 2012 (Fig. 2). Every month, glass slips with 1-, 2-, 3- and 4-week-old biofilms were removed from the PVC holders and placed in a container with GF/C (Whatman glass fiber filter; pore size: 1.2 μm) filtered seawater (FSW) to prevent the biofilms from drying up. Biofilms were brought back to the laboratory for microscopic observation and quantitative analyses. For any quantitative measurement taken each month, data obtained from biofilms of the same immersion period were averaged, so that there was only 1 replicate measurement each for the 1-, 2-, 3- and 4-week-old biofilms every month.

Seawater temperature and salinity of the study site were obtained from data published by the Nagasaki Prefectural Institute of Fisheries (2011; 2012; 2013). Values were from measurements taken at the sea surface (<1 m depth) of the study site.

Enumeration of periphytic diatoms and other fouling communities attached on glass slips

Glass slips with the biofilms were immediately observed under a light microscope to enumerate periphytic diatoms and verify other fouling communities attached on the glass slips. Identification of diatoms to the genus level was conducted every month but only on biofilm samples with the highest diatom densities in each month. The purpose was to identify the composition of the diatom communities in established biofilms. Diatoms were classified to the genus level based on illustrated manuals of marine and benthic diatoms of Japan (Yamaji, 1984; Kawamura, 1994). Diatoms in the biofilms were counted under a light microscope at 200× and 400× magnifications from 10 random fields of view from 1 or 2 glass slips.

Measurement of dry weight and chlorophyll contents of biofilms

Dry weights of the biofilms and fouling communities on glass slips were measured following the method employed...
Growth of marine biofilms
by Bao et al. (2007b). Biofilms and fouling communities on
glass slips were scraped off using a clean glass slip and were
suspended in FSW. Each suspension was collected on a pre-
weighed glass filter paper GF/C (ϕ 47 mm) by filtration. The
sample on the GF/C was then desalted by filtering 50 mL of
DW. GF/C with the samples on it were then dried in an oven
at 80°C for 2 hr and cooled to room temperature in a desicca-
tor before weighing. Dry weights of 1-week-old biofilms were
collected from 2 to 10 glass slips immersed in the sea for 1
week, while dry weights of each of 2-, 3- and 4-week-old bio-
films were collected from 2 to 6 glass slips immersed in the
sea for 2, 3 and 4 weeks, respectively.

Biofilms and fouling communities were also collected on
GF/C to measure the chlorophyll contents of the biofilms.
Chlorophyll contents of 1-week-old biofilms were estimated
from 2 to 18 glass slips immersed in the sea for 1 week, while
chlorophyll contents of each of the 2-, 3- and 4-week-old bio-
films were estimated from 2 to 6 glass slips immersed in the
sea for 2, 3 and 4 weeks, respectively. Chlorophyll contents
of biofilms were extracted with 90% (v/v) acetone (Nakarai
Tesque). Chlorophyll contents were determined after mea-
suring absorbance of the extracts at 750 nm, 664 nm, 647 nm
and 630 nm using a Hitachi U-1900 spectrophotometer.
Chlorophylls-α, -β, and -c contents were calculated using the
equation of Jeffrey and Humphrey (1975). Chlorophylls were
expressed either as the amount in µg per unit area (µg cm$^{-2}$)
dry weight of the biofilm.

Statistical analysis
Correlations between the total dry weight of biofilms and
the amount of chlorophyll (α, β and c) per unit glass area
were analyzed using a Pearson correlation test in order to
check whether chlorophyll can be used as a valid index of
biofilm biomass. The individual effects of immersion time
(1, 2, 3 and 4 weeks) and season (Jan to Mar, Apr to May,
Jun to Aug and Sep to Dec) and the interaction effect of these
two factors on the chlorophyll contents and diatom density
of biofilm during development were analyzed using two-way
ANOVA, after data were pooled together according to immer-
sion periods and seasons. Replicates for each of the immer-
sion periods 1, 2, 3 and 4 weeks were 20–22, 26–28, 25–27
and 29–30, respectively, and replicates for the seasons of Jan
to Mar, Apr to Jun, Jul to Sep and Oct to Dec were 21–22,
24–25, 27–34 and 27, respectively. Seasonal variations in
algal biomass (expressed as chlorophyll contents) and diatom
densities of biofilms were also assessed by post hoc Tukey
HSD multiple comparison tests. All statistical computations
were performed using JMP ™ software. Differences were
considered significant at $p<0.05$.

Results
The temperature and salinity (10-day intervals) of the
seawater at the study site and the biomass (in mg cm$^{-2}$ dry
weight) of biofilms and fouling communities on the glass slips
submerged at the same area during the period from July 2010
to October 2012 are shown in Fig. 3. During the 28-month
study period, monthly average seawater temperatures at the
study site were from 12 to 14 °C between Jan and Mar, from
16 to 22 °C between Apr and May, from 25 to 28 °C between
Jul and Sep, and from 17 to 23 °C between Oct and Dec. Throughout the study period, the monthly average salinity of
seawater at the study area was between 30 and 34.

Fig. 3. Water temperatures and salinities at 10-day intervals, and the dry weights of marine biofilms and other fouling communities on glass
slips submerged for 1, 2, 3 and 4 weeks every month in the sea off Nagasaki Prefectural Institute of Fisheries from Jul 2010 to Oct 2012. Water
temperatures and salinities were from data published by Nagasaki Prefectural Institute of Fisheries (2011; 2012; 2013).
Total dry weights (mg cm\(^{-2}\)) of biofilms and fouling communities on the glass slips

In general, total dry weights of biofilms and fouling communities on the glass slips increased with longer immersion periods (Fig. 3). During the months from Jun to Aug, when sea water temperatures ranged between 21 and 29 °C, invertebrate macrofoulers were observed attached on the glass slips, even on those immersed for only 2 weeks. Invertebrate macrofoulers that attached on the glass slips during these months were the polychaete *Dexiospira foraminosa*, amphipods (Gammaridae) and the barnacles *Amphibalanus amphitrite* and *Balanus trigonus*. Individual numbers of these invertebrates were not counted. During these months, maximum total dry weights of fouling communities reached 3 mg cm\(^{-2}\) in 2010, 35 mg cm\(^{-2}\) in 2011, and 6 mg cm\(^{-2}\) in 2012. By contrast, no invertebrate macrofoulers attached on glass slips immersed during the months from Sep to May, except in Sep 2011 where glass slips had polychaetes and barnacles attached. In the months where glass slips had no invertebrate macrofoulers attached, total dry weights of 4-week-old biofilms ranged from 0.3 (Sep 2010) to 1.2 mg cm\(^{-2}\) (Feb 2011).

Biofilms that developed on the glass slips consisted mainly of diatoms. Young thalli of *Ulva compressa* also occurred in the biofilms from Apr to Dec, although it was not quantified.

**Chlorophyll contents as an index of marine biofilm biomass**

Dry weight has been used as a conventional index for measuring the growth of biofilm biomass (e.g. Chiu et al., 2005; Bao et al., 2007b). The correlations between the total dry weight of biofilms and the amounts of chlorophylls (\(-a\), \(-b\) and \(-c\)) per unit area of glass slip in the different seasons are shown in Fig. 4. Amounts of chlorophylls \(-a\) and \(-c\) per unit area of glass slips both showed positive correlations with the total dry weight of biofilms in all the four seasons (Jan–Mar, Apr–Jun, Jul–Sep, Oct–Dec), indicating that both chlorophylls \(-a\) and \(-c\) are also valid indices of marine biofilm biomass. By contrast, no correlation was observed between the amount of chlorophyll \(-b\) per unit area of glass slip and the total dry weight of biofilms in the months of Apr to Jun (\(r=0.06, P>0.05\)) and Jul to Sep (\(r=0.05, P>0.05\)), suggesting that chlorophyll \(-b\) may not be an accurate index for estimating the variation in biomass of marine biofilms.

Chlorophylls \(-a\) and \(-c\) contents (in µg mg\(^{-1}\)) of biofilms on the glass slips submerged during the study period are shown in Figs. 5 and 6. Due to the complex micro-topographies of older biofilms, which included patches composed of more than one layer of epiphytic microalgae, chlorophylls \(-a\) and \(-c\) contents were expressed in relation to the weight of biofilms instead of the surface area of glass slips.

In general, chlorophyll \(-a\) content of biofilms reached maximum values in 2 to 3 weeks after immersion of glass slips, with values either remaining constant or gradually declining on the 4th week of immersion. Monthly average chlorophyll \(-a\) contents ranged from 2.5 to 5.7 µg mg\(^{-1}\) total dry weight of biofilm, with the lowest values measured during the months of Jul to Sep (Fig. 5). The maximum chlorophyll \(-a\) content was 13.3 µg mg\(^{-1}\) of biofilm, measured in Jun of 2011 in 3-week-old biofilms.

Chlorophyll \(-c\) contents of biofilms generally reached
maximum values after 2 to 3 weeks of immersion from Jan to Apr and Oct to Dec. Monthly average chlorophyll-c contents ranged from 0.3 to 0.9 µg mg⁻¹ total dry weight, and a maximum chlorophyll-c content of 1.8 µg mg⁻¹ of biofilm was measured in Jun of 2011 in 3-week-old biofilms (Fig. 6).

Analysis of variance showed that chlorophyll-a contents of biofilms significantly varied with immersion period ($P=0.0158$) and season ($P<0.0001$), although no interaction was seen between immersion period and season for the pigment ($P=0.1388$), indicating that immersion period-season interaction had no significant effect on the variation in chlorophyll-a contents of biofilms (Table 1). On the other hand, chlorophyll-c contents of biofilms did not significantly vary with the immersion period ($P=0.1695$) but varied with the seasons ($P=0.0004$, Table 1). Immersion period-season interaction also had no significant effect on the variation in chlorophyll-c contents of biofilms ($P=0.1396$, Table 1). Post hoc Tukey HSD multiple comparison test showed that biofilms had significantly higher chlorophylls-a and -c contents from Oct to Mar as compared to those from Jul to Sep (Table 2).
Abundance and composition of periphytic diatom communities in biofilms

Diatom densities in biofilms of different immersion periods and from different months are shown in Fig. 7. In general, diatoms in the biofilms reached maximum densities after 2 to 4 weeks of immersion of glass slips. Monthly average diatom densities were 2.4 to 3.6×10^5 cells cm^{-2} from Jan to Mar, 2.4 to 2.7×10^5 cells cm^{-2} from Apr to Jun, 1.0 to 1.9×10^5 cells cm^{-2} from Jul to Sep, and 2.8 to 3.3×10^5 cells cm^{-2} from Oct to Dec (Fig. 7). Analysis of variance showed that the abundance of diatoms in the biofilms was significantly affected by the immersion period (P<0.0001) and season (P=0.0001, Table 1). Moreover, the interaction between the season and immersion period significantly affected the variation in diatom density of biofilms (P=0.0268); indicating that the effect of immersion period on the abundance of diatoms in the biofilms was modulated by the seasons (Table 1). Post hoc Tukey HSD multiple comparison test showed that average diatom densities were significantly lower from Jul to Sep as compared to the other months (Table 2).

Table 3 shows the occurrence of periphytic diatoms in biofilms collected every month during the 28-month study period. Diatoms were identified to the genus level only in the biofilm sample with the highest diatom density in each month. Throughout the study period, *Navicula* and *Nitzschia* occurred in all biofilm samples. Except in the Jan 2012 sample, *Cocconeis* was dominant in Aug 2011 and also from May to Aug of 2012. *Cocconeis* was dominant in Aug 2011 and also from May to Aug of 2012. *Nitzschia* was

Table 1. ANOVA results of the effect of immersion period and season on chlorophyll-a and -c contents, and the density of diatoms.

| Factor                | Level                          | Chlorophyll-a | Chlorophyll-c | Density of diatoms |
|-----------------------|--------------------------------|---------------|---------------|-------------------|
|                       | df   | F     | P     | df   | F     | P     | df   | F     | P     |
| Immersion period (weeks) | 3    | 3.631 | 0.0158 | 3    | 1.7181 | 0.1695 | 3    | 21.4187 | <0.0001 |
| Season                | Jan–Mar, Apr–Jun, Jul–Sep, Oct–Dec | 3 8.0217 0.0001 | 3 6.6552 0.0004 | 3 7.7105 0.0001 |
| Week×Season           | 9    | 1.5615 | 0.1388 | 9    | 1.5638 | 0.1396 | 9    | 2.2306 | 0.0268 |

For 1, 2, 3 and 4 weeks of immersion period, the number of replicates were 20–22, 26–28, 25–27 and 29–30, respectively. For the seasons of Jan–Mar, Apr–Jun, Jul–Sep and Oct–Dec, the number of replicates were 21–22, 24–25, 27–34 and 27, respectively.

Table 2. Chlorophyll-a and -c contents, and the density of diatoms in Jan–Mar, Apr–Jun, Jul–Sep, and Oct–Dec.

|                      | Jan–Mar | Apr–Jun | Jul–Sep | Oct–Dec |
|----------------------|---------|---------|---------|---------|
| Chlorophyll-a (µg mg^{-1}) | 5.74±2.36 (21) a | 3.82±3.10 (25) bc | 2.49±1.72 (34) c | 5.12±1.99 (27) ab |
| Chlorophyll-c (µg mg^{-1}) | 0.87±0.39 (21) a | 0.48±0.41 (25) bc | 0.34±0.26 (27) c | 0.64±0.37 (27) ab |
| Density of diatoms (×10^5 cells cm^{-2}) | 3.05±1.68 (22) a | 2.61±1.53 (24) a | 1.57±0.94 (34) b | 3.05±1.59 (27) a |

Values are means±standard deviation of all data obtained in each season. Numbers in parentheses indicate the number of replicates. Letters indicate results of the post hoc Tukey HSD test. For each item, groups connected by the same letter are not significantly different (p≥0.05).
also dominant from Aug to Sep 2010, from Apr to May 2011, and in Apr, May and Oct 2012. Amphora occurred in biofilms from Oct to Dec in 2010, from Jan to Mar and Oct to Dec in 2011, and from Jan to Mar and Jun to Sep in 2012. Bacillaria occurred only in biofilms in Oct 2010 and from Jan to Mar 2012, while Rhizosolenia occurred in biofilms from Oct to Nov 2010, Oct 2011, and Feb and Apr of 2012. Both Bacillaria and Rhizosolenia comprised <1% of diatom assemblages in biofilms where they occurred.

### Discussion

Studies on biofilm development on submerged surfaces in the marine environment usually focus on biofilm growth during a period of 1 to 14 days (e.g. Nayar et al., 2005; Chiu et al., 2008; Satheesh and Wesley, 2012). However, biofilms have a dynamic structure and composition, and even mature biofilms are continuously changing, reflecting the environmental factors that are affecting the substratum (Patil and Anil, 2005; Qian et al., 2007). Therefore, studying biofilm growth for longer time periods would provide a more realistic timeframe for biofilm development (Campbell et al., 2011).

In the present investigation, glass slips that were immersed in the sea acquired biofilms. These biofilms consisted mainly of diatoms and young thalli of Ulva compressa (Apr to Dec), and their mass (in dry weight) generally continued to increase with the immersion period of up to 4 weeks (Fig. 3). This result justifies the necessity to look into biofilm development in the marine environment on a longer timeframe, and that is weeks instead of days. During the months from Jun to Sep, however, invertebrate macroorganisms also attached on the glass slips in addition to biofilms, such that the bulk of the dry weights measured during these months were those of the invertebrate macroorganisms. Consequently, the estimation of the dry weight in order to evaluate growth of biofilms during these months may not be appropriate, if not difficult. Previous studies evaluated the development of marine biofilms by the direct method of observing and counting diatom composition and abundance (Patil and Anil, 2005; Mitbavkar and Anil, 2008; Satheesh and Wesley, 2012) and/or by measuring the chlorophyll contents (Patil and Anil, 2005; Mitbavkar and Anil, 2008) of biofilms.

Chlorophyll-\(a\) is present in all photosynthetic algae (Dere et al., 1998), and is used as an index to estimate algal biomass in the water column (Steele, 1962; Cullen, 1982), sediments (Leach, 1970; Joint, 1978; Lukatelich and McComb, 1986) and biofilms (Patil and Anil, 2005). However, Ramaraj et al. (2013) argued that chlorophyll is not an accurate index for algal biomass, based on their findings that direct measurement of the dry weight and the chlorophyll content of cultured algae showed no apparent relationship in any of the statistical models they tested. Our study demonstrated a linear regression between the amount of chlorophyll-\(a\) and -\(c\) and the dry weight of biofilms, indicating that chlorophylls-\(a\) and -\(c\) are valid indices of estimating algal biomass in biofilms (Fig. 4). However, there was no discernible relationship found between the amount of chlorophyll-\(b\) and the dry weight of biofilms (Fig. 4). Considering that the biofilms that developed on the glass slips consisted mainly of diatoms, the linear regres-

**Table 3.** Occurrence of periphytic diatoms in biofilm samples with the highest diatom density in each month from Jul 2010 to Oct 2012.

| Diatoms (Genus) | Year | Jan | Feb | Mar | Apr | May | Jun | Jul | Aug | Sep | Oct | Nov | Dec |
|----------------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Amphora        | 2010 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2011 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2012 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Bacillaria     | 2010 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2011 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2012 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Cocconeis      | 2010 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2011 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2012 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Navicula       | 2010 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2011 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2012 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Nitzschia      | 2010 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2011 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2012 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Rhizosolenia   | 2010 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2011 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
|                | 2012 | ±   |    | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |

Abundance of each genus: ± indicates <1% of the total diatom count; +, 1–24% of total diatom count; ++, 25–50% of total diatom count; and ++++, >50% of total diatom count. Monthly data were taken from samples with the highest diatom density of each month. Horizontal bars indicate the periods that were not included in the present study.
sion found between chlorophyll (-a and -c) and the dry weight of the biofilm would be a predictable consequence.

Chlorophyll-a contents of the biofilms varied with the immersion period (Fig. 5, Table 1) but no temporal variation was observed in the chlorophyll-c contents of the biofilms (Fig. 6, Table 1). Ritchie (2008) cautioned that when the abundance of chlorophyll-c compounds is low, chlorophyll-c values tend to be overestimated. This may explain why chlorophyll-c contents did not vary with the immersion period; actual chlorophyll-c contents of young biofilms may have been underestimated. Nevertheless, this warrants further investigation.

Previous studies on benthic microalgae in intertidal systems (e.g. Leach, 1970; Joint, 1978) and on biofilms (e.g. Chiu et al., 2005; Mitbavkar and Anil, 2008) have reported seasonal variation in algal biomass. The present study also encountered seasonal variations in the chlorophylls-a and -c contents of the biofilms (Figs. 5, 6, Table 1). Chlorophylls-a and -c contents of biofilms were higher in the months of Oct to Mar when average monthly water temperatures ranged from 12 to 23°C, as compared to those in Jul to Sep when average monthly water temperatures ranged from 25 to 28°C and the attachment of invertebrates occurred (Table 2). Seasonal variation in chlorophylls-a and -c contents of biofilms in this study may be attributed to the possible disturbance of the biofilm community by invertebrate macrofoulers that occurred in Jun to Sep, although no direct observation was conducted on the activities of macrofoulers on the biofilms, thus warranting further investigation.

The present study also found temporal and seasonal variations in the abundance of diatoms in the biofilm, and these two factors interacted to affect the abundance of diatoms in the biofilm (Fig. 7, Table 1). This finding is in accordance with the temporal and seasonal variations in chlorophyll-a contents of biofilms observed in this study. Average monthly densities of diatoms in biofilms were constant but decreased in Jul to Sep, probably due to disturbance of the biofilm by the attachment of invertebrates during this period (Table 2). Previous studies attributed seasonal variations in diatom densities of marine biofilms on physico-chemical (e.g. temperature and salinity (Patil and Anil, 2005; Chiu et al., 2005)) and biological (e.g. grazing pressure from amphipods (Kawamura and Hirano, 1992)) factors. These factors may have also contributed to the seasonal variation observed in the diatom densities of the biofilms in this study.

Diversity of diatom communities in the biofilms also exhibited seasonal variations; diatom communities were more diverse during the months from Oct to Mar than between Apr and Sep, with 4 to 6 genera occurring in the former, while only 3 to 4 genera were observed in the latter (Table 3). This finding is consistent with other studies (Patil and Anil, 2005; Mitbavkar and Anil, 2008), which also reported seasonal variations in the diatom community structure of biofilms, even though the present study observed diatom community compositions in established biofilms which were >3 weeks old, while previous reports studied 4-day-old biofilms (Patil and Anil, 2005; Mitbavkar and Anil, 2008). Seasonal variation in the diversity of diatom communities in the biofilms may be attributed to the difference between species in their tolerance to the effect of grazing (Suzuki et al., 1987), which becomes intense in the summer (Kawamura and Hirano, 1992). Moreover, Navicula was almost always the dominant diatom in the established biofilms, except in certain months between May and Aug when either Nitzschia or Cocconeis occurred with Navicula as dominant diatoms (Table 3). Satheesh and Wesley (2012) also reported that Navicula and Nitzschia were the dominant diatoms observed throughout their one year study.

Measuring dry weight (e.g. Chiu et al., 2005; Bao et al., 2007b) or the abundance of bacteria (e.g. Bao et al., 2007b) and diatoms (e.g. Satheesh and Wesley, 2012) has been the conventional method of estimating biofilm growth on submerged surfaces in the marine environment. However, while dry weight may not be an accurate index for measuring variation in biomass of biofilms with macrofoulers in it, direct counting of bacteria and diatoms in biofilms may often be difficult because micro-topographies of biofilms become more complicated with longer immersion periods. The present study confirmed that chlorophyll content is a valid index for measuring temporal and seasonal variation in the biomass of marine biofilms that are mainly composed of periphytic microalgae. Nevertheless, simpler yet definitive methods for quantitative assessment of marine biofilm growth still need to be developed.

Acknowledgement

The authors wish to express their gratitude to Mr. T. Fukase for extending technical assistance. The authors also thank Nagasaki Prefectural Institute of Fisheries for allowing the use of their pontoon raft.

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