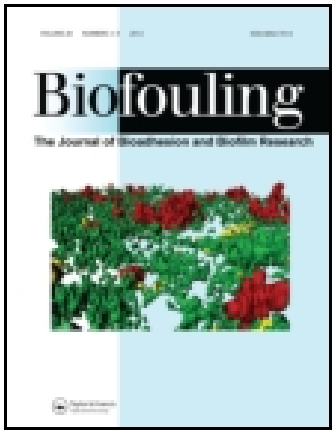

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An approach to determining the sampling effort for analyzing biofilm-dwelling ciliate colonization using an artificial substratum in coastal waters

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A new approach to determining sampling effort for analyzing biofilm-dwelling ciliate colonization was studied in the coastal waters of the Yellow Sea, northern China, from May to June 2010. The optimal sample size for evaluating biofilm-dwelling ciliate colonization increased with shortening exposure time, and can be determined according to the probability of recovering those species with a specified cumulative contribution to communities. More slide-replicates were required at a depth of 3 m than at 1 m to recover equivalent proportions of the ciliate communities. For routine colonization dynamics analyses, 10 slide-replicates (175 cm^2) were sufficient to achieve a 95% probability of recovering those species with a cumulative contribution of >90% to the ciliate communities at a depth of 1 m. These results suggest that 10 slide-replicates immersed at a depth of 1 m may be an optimal sampling strategy for analyzing the colonization dynamics of biofilm-dwelling ciliate communities in marine habitats.

Keywords: biofilm-dwelling ciliate; sampling effort; colonization dynamics; artificial substratum; field community; marine ecosystem

Introduction

Biofilm-dwelling ciliates are a primary component of the periphyton or *aufwuchs* communities in many aquatic ecosystems and play an important role in the functioning of microbial food webs by mediating the flux of organic matter and energy from the plankton to the benthos in many aquatic ecosystems (Bryers and Characklis 1982; Eisenmann et al. 2001; Fischer et al. 2002; Weitere et al. 2003; Kathol et al. 2009; Norf et al. 2009a, 2009b). They are also the primary contributors in maintaining and improving water quality by removing organic matter (Cairns and Henebry 1982; Foissner and Berger 1996; Madoni 2003; Dubber and Gray 2009). Furthermore, with their rapid responses to environmental changes, ease of sampling, relative immobility, increasing availability of user-friendly taxonomic references and standardized methodologies for temporal and spatial comparisons, periphytic ciliates have widely been accepted as robust indicators to evaluate environmental stress and anthropogenic impacts in aquatic ecosystems (Morin et al. 2008; Risse-Buhl and Küsel 2009; Xu et al. 2009a, 2009b; Mieczan 2010).

The operational characteristics (eg species richness and abundance) of ciliate communities are associated with a wide range of physico-chemical parameters which in turn determines the amount of sampling effort or sample sizes required (Masseret et al. 1998; Xu et al. 2005; Jiang et al. 2007; Norf et al. 2009a; Morin et al. 2010). A variety of approaches based on artificial substrata for collecting periphytic communities have previously been used, particularly in terms of the number of replicates and the depths of exposure (Railkin 1998; Strüder-Kypke 1999; Kralj et al. 2006; Norf et al. 2007; Xu et al. 2009a). However, optimizing the sampling effort for analyzing ciliate colonization features has received comparatively little attention (Weitere et al. 2003; Norf et al. 2009a, 2009b; Xu et al. 2009a, 2009b).

A 1-month baseline survey of ciliate colonization processes was conducted using an artificial substratum (glass slides) in the coastal waters of the Yellow Sea, near Qingdao, northern China during May and June 2010. The aims were: (1) to determine the optimum sampling effort for evaluating the temporal dynamics of the ciliate colonization, and (2) to determine the optimal sample size for routine analyses of the ciliate

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communities during different exposure periods at different depths.

Materials and methods

Study site and sampling strategy

The study was conducted in the coastal waters of the Yellow Sea, near the Olympic Sailing Center at Qingdao, northern China from 18 May to 16 June 2010 (water temperature 14–19°C; pH ~8; salinity 30–31 psu; dissolved oxygen concentration 6–7 mg l^{-1}). This coastal area is ~8 m deep with sunlight able to penetrate to a depth (transparency) of ~3 m (Figure 1).

The glass slide systems were designed, deployed, anchored, and sampled as described by Xu et al. (2009a, 2009b). A total of 280 glass slides (2.5 cm × 7.5 cm) were used as artificial substrata for collecting biofilm-dwelling ciliates at depths of 1 m and 3 m below the water surface. For each depth, a total of seven PVC frames were used to hold a total of 140 slides, 20 of which were randomly collected from each PVC frame at each of the following colonization times: 1, 3, 7, 10, 14, 21 and 28 days. Samples were collected simultaneously from both depths.

Identification and enumeration

Ciliate identification and enumeration were conducted following the methods outlined by Xu et al. (2009a, 2009b). Protargol staining was performed for species identification (Pan et al. 2010). Taxonomic classification of ciliates was based on published keys and guides such as Song et al. (2009), Fan et al. (2010) and Jiang et al. (2010).

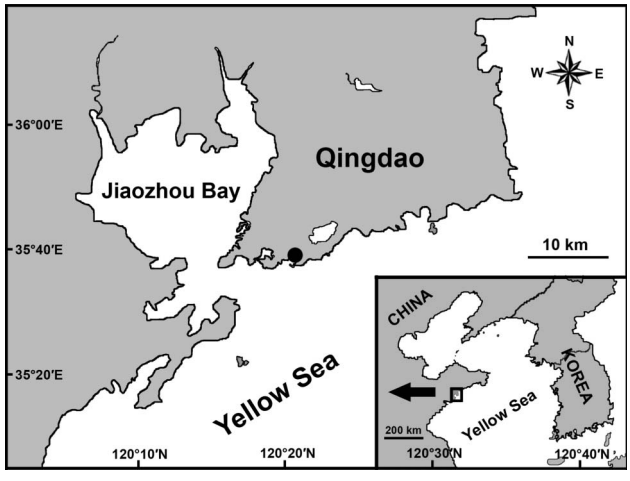

Figure 1. Map showing the site of the sampling station, which was located on the Yellow Sea coast near Qingdao, northern China.

The enumeration of ciliates *in vivo* was conducted at a 100× magnification under an inverted microscope as soon as possible after sampling (generally within 24 h) in order to prevent significant changes in species number and composition (Xu et al. 2009a, 2009b). In order to recover all species colonizing the glass slides, one surface of an entire slide (17.5 cm^2) from a total of 40 slide-replicates was examined at each colonization period using bright field illumination and occurrences were recorded. For the enumeration of individual abundances, one entire slide surface was examined at each colonization period of <14 days, whereas for colonization period of 14 days or more, 10 randomly chosen fields of view per slide were examined and the dominant ciliates were enumerated. The ciliate abundances were calculated for 20 sample sizes with 1–20 slide-replicates to confirm the average abundance of ciliate individuals (individuals cm^{-2}).

Data analyses

The probability P of finding all species expected was calculated following the equation:

$$P = [1 - (1 - p_1)^n][1 - (1 - p_2)^n] \ldots [1 - (1 - p_k)^n]$$

where p is the probability of success of recovering a species, k is the number of species recovered, and n is the number of replicates of glass slides (Dubber and Gray 2009).

Species diversity (H'), evenness (J') and species richness (D) of samples were calculated as follows:

$$H' = -\sum_{i=1}^{S} P_i(\mathrm{In}\, P_i)$$

$$J' = H'/\mathrm{In}S$$

$$D = (S - 1)/\mathrm{In}N$$

where H' = the observed diversity index, P_i = the proportion of the total count arising from the ith species, S = the total number of species, and N = the total number of individuals.

The colonization process of the ciliate communities can be followed by sampling replicate glass slides over time to follow the rate and magnitude of each species accrual process. Data can thus be fitted to the colonization equilibrium model developed by MacArthur and Wilson (1967):

$$S_t = S_{\mathrm{eq}}\,(1 - e^{-Gt})$$

where S_t = the species number at time t, S_{eq} = the estimated equilibrium species number of ciliate

colonization, G = the colonization rate constant, and $T_{90\%}$ = the time taken for reaching 90% S_{eq}. Three colonization parameters (S_{eq}, G, and $T_{90\%}$) were estimated using the SIGMAPLOT. Fitness tests were conducted to determine whether the species numbers observed at each time interval fit with the MacArthur–Wilson model at the 0.05 significance level.

The three functional parameters (S_{eq}, G, and $T_{90\%}$) have been wildly used as a robust means of predicting the loading capacity or assimilative capacity of an aquatic ecosystem for contaminant inputs (Xu et al. 2002). Among these, the S_{eq} is commonly negatively associated with concentrations of organic pollutants and levels of toxicity, whereas the G value is generally high in the waters with lower environmental stress (Cairns and Henebry 1982).

Boxplots were plotted to summarize the variations in structural parameters (number, abundance and diversity of species) of biofilm-dwelling ciliate communities with different sample sizes by whiskers (minimum and maximum), boxes ($\pm$ 25%) and lines (medians) (Hansson et al. 1993).

The coefficient of variation (CV) was used to show the standard errors of biotic parameters of the ciliate communities among the different sampling regimes. Contour plots with scale-values were constructed for visualizing these CV values in the scales (eg 5%–50%).

The nonparametric Kolmogorov–Smirnov test was used to evaluate the differences in colonization curves of ciliate species among different sample sizes at the 0.05 level.

All multivariate analyses and routines were conducted using PRIMER v6.1 (Clarke and Gorley 2006). The similarities of the colonization patterns of ciliate communities based on different sampling regimes were analyzed by the second-stage MDS ordination on the second-stage similarity matrix from the first-stage Bray–Curtis similarity matrices using the 2STAGE routine. The contributions of ciliate species to the ciliate communities were evaluated using the SIMPER routine (Clarke and Gorley 2006). The original species-abundance data were fourth-root transformed before analyses.

Results

Expected sampling efforts according to both occurrences and contributions of ciliate species

The occurrences of the ciliates in 1-, 3-, 7-, 10-, 14-, 21- and 28-day communities with 20 replicates at the two depths (1 m and 3 m) in the coastal waters of the Yellow Sea during the study period are summarized in Table 1. A total of 80 ciliate species were recovered from both depths over the whole survey period, of which 74 and 65 species were found at depths of 1 m and 3 m, respectively (Table 1). SIMPER analyses demonstrated that a total of 55 species are the 'typical' species with a cumulative contribution of 99.5% to ciliate communities within different colonization periods (shaded grey in Table 1). It should be noted that the occurrences of ciliates based on 20 slide-replicates increased with the duration of exposure time. For example, the ciliate *Diophrys appendiculata* and *Tachysom dragescoi* occurred in the ciliate communities with relatively low frequencies (5%–65%) during 1–7 day periods, but with significantly higher frequencies (95%–100%) after 10 days (Table 1).

The numbers of slide-replicates required to achieve a 95% probability of recovering those species with a cumulative contribution of 90% to ciliate communities varied according to colonization period (Table 2). For example, in order to recover those species with a cumulative contribution of 95% after $>$7-day colonization, 3–10 slide-replicates were required. However, this rose to 13–19 slide-replicates for colonization periods of 3–7 days (Table 2). For higher cumulative contributions, significantly more slide-replicates were needed. For example, at colonization times of $>$7 days, 11–20 and 88–103 slide-replicates were needed in order to recover those species contributing 99.5% and 100% respectively of the ciliate communities (Table 2). It is also noteworthy that at colonization times of $>$1 day, 10 and 20 slide-replicates were sufficient to recover those species with cumulative contributions of more than 90% and 95%, respectively (Table 2).

Sampling effort required for evaluating species number and abundance

The species numbers and abundances of ciliates in 1-, 3-, 7-, 10-, 14-, 21- and 28-day communities based on 1–20 slide-replicates at the two depths during the study period are shown in Figures 2a, b and 3a, b. The boxplots reveal that the ciliate species numbers and abundances of ciliates increased as the number of slide-replicates increased and water depth decreased (Figures 2a, b and 3a, b). It should be noted that the abundances in 28-day samples at the depth of 1 m sharply dropped to a low level.

The coefficient of variation (CV) values in both species numbers and abundances of the ciliate communities at the two depths over the study cycle among the samples based on 1–20, 2–20, 3–20, ... , and 19–20 slide-replicates are summarized in Figures 2c, d and 3c, d. These demonstrate that the CV values in terms of both species number and abundance show a clear increasing trend with the decrease of slide-replicates and the increase of exposure water depths at each colonization time (Figures 2c, d and 3c, d). For

Table 1. Occurrences of periphytic ciliates in 1-, 3-, 7-, 10-, 14-, 21- and 28-day communities with 20 replicates at two depths (1 m and 3 m) at the study site during the study period.

	1-day		3-day		7-day		10-day		14-day		21-day		28-day	
Species	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m
Pseudovorticella sp.1	10	10	15	10	90	70	100	100	100	100	100	100	100	100
Aspidisca aculeata	5	–	50	20	85	60	100	95	100	100	100	90	95	100
Tachysoma dragescoi	5	–	35	10	65	65	100	100	100	100	100	90	100	100
Litonotus paracygnus	–	5	35	5	85	40	100	95	100	100	95	65	95	100
Tachysoma ovata	–	–	–	–	60	25	100	80	100	100	100	95	100	50
Euplotes rariseta	–	–	15	5	10	–	70	50	50	65	60	60	100	75
Litonotus yinae	–	–	–	–	30	5	65	30	100	90	90	95	65	25
Diophrys appendiculata	–	–	5	–	5	10	75	35	100	100	100	100	100	100
Litonotus songi	–	–	–	–	5	10	100	55	95	85	75	55	85	40
Orthodonella sp.	–	–	–	–	25	–	30	5	100	75	100	95	100	100
Spirostrombidium cinctum	–	–	–	–	–	–	70	30	95	90	100	95	70	30
Folliculina simplex	–	–	10	5	20	10	30	5	40	30	100	70	35	100
Dysteria derouxi	–	–	–	–	30	15	100	75	90	95	15	5	45	–
Dysteria pusilla	–	–	–	–	20	–	65	40	85	35	55	80	–	10
Anteholosticha warreni	10	–	20	–	15	30	10	–	40	25	–	–	35	–
Coeloperix sleighi	–	–	–	–	–	–	–	–	75	85	90	50	55	70
Zoothamnium plumula	–	–	5	–	10	15	15	20	20	30	80	10	10	90
Paracineta sp.	–	–	–	–	–	10	20	–	35	80	100	80	85	70
Apotrachelotractus variabialis	–	–	5	–	–	–	15	15	65	80	85	70	5	85
Euplotes vannus	5	5	25	5	5	15	35	5	30	5	5	30	70	35
Hartmannula angustipilosa	–	–	–	–	10	10	85	40	60	85	15	20	20	5
Conchacineta complatana	–	–	–	–	–	5	30	35	10	15	5	10	75	45
Protocruzia contrax	–	–	–	–	20	–	35	25	15	15	40	30	75	5
Trochilia sp.	–	–	–	–	30	5	90	60	25	75	5	10	20	–
Ephelota truncata	–	–	–	–	–	–	5	5	5	25	100	75	100	100
Stephanopogon minuta	–	–	–	–	5	–	75	35	35	55	5	–	65	–
Euplotes raikovi	–	–	–	–	–	–	–	–	–	20	80	80	95	95
Certesia quadrinucleata	–	–	5	–	10	–	25	20	65	65	5	5	5	–
Holosticha diademata	–	–	–	–	5	–	25	15	–	20	85	35	95	95
Aspidisca leptaspis	–	–	–	–	–	–	–	20	15	15	40	20	85	30
Condylostentor auriculatus	–	–	–	–	–	–	5	–	50	25	90	35	15	100
Litonotus sp.2	–	–	–	–	–	–	–	–	40	40	25	15	25	40
Paralembus digitiformis	–	–	–	60	–	25	–	20	–	–	–	35	20	25
Strombidium sulcatum	–	–	–	–	–	5	40	15	70	65	10	–	–	–
Parauronema longum	–	–	55	60	10	10	–	–	–	–	–	–	–	–
Pseudovorticella sp.3	–	–	–	35	–	–	30	–	–	–	–	–	90	–
Frontonia tchibisovae	–	–	–	5	–	–	5	10	15	15	80	5	5	90
Holosticha heterofoissneri	–	–	–	10	–	–	–	–	5	–	85	20	35	–
Uronemella sp.	5	–	–	–	40	60	–	–	–	–	–	–	–	–
Amphileptus houi	–	–	5	5	10	–	5	5	25	5	20	10	45	30
Acineta tuberosa	–	–	–	–	25	5	25	5	10	–	10	10	15	–
Lacrymaria marina	–	–	–	–	5	–	–	5	5	–	55	5	–	15
Lacrymaria maurea	–	–	–	–	–	–	5	10	15	–	90	30	–	5
Pseudokeronopsis flava	–	–	–	–	–	–	5	–	30	30	–	–	10	100
Hartmannula derouxi	–	–	–	–	5	–	30	–	–	25	–	–	–	–
Thuricola sp.	–	–	–	–	–	–	–	–	–	5	5	–	50	–
Dysteria crassipes	–	–	5	–	–	–	30	–	–	–	–	–	–	–
Oxytricha saltans	–	–	–	–	–	–	5	–	–	–	–	–	65	–
Trachelostyla pediculiformis	–	–	–	–	–	–	–	–	–	–	30	–	–	75
Loxophyllum choii	–	–	–	–	–	–	–	–	–	–	–	–	55	–
Metaurostylopsis salina	–	–	20	–	–	–	–	–	–	–	–	–	–	–
Miamiensis avidus	–	–	10	–	–	–	–	–	–	–	–	–	–	–
Pseudoamphisiella elongata	–	–	–	–	–	–	–	–	–	–	–	–	–	40
Pseudovorticella verrucosa	–	–	30	–	–	–	–	–	–	–	–	–	–	–
Strombidium conicum	–	–	20	–	–	–	–	–	–	–	–	–	–	–
Psammomitra retractilis	–	–	–	–	–	–	15	15	10	15	5	–	5	–
Uronychia sp.	–	–	–	–	–	–	–	–	25	–	30	30	10	5
Paradiscocephalus elongatus	–	–	–	–	–	–	–	–	–	10	5	5	5	5
Acineria incurvata	–	–	–	–	–	5	–	–	–	–	5	–	5	5
Licnophora lyngbycola	–	–	–	–	–	–	–	–	–	5	5	–	5	20

(*continued*)

Table 1. (*Continued*).

Species	1-day		3-day		7-day		10-day		14-day		21-day		28-day	
	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m
Pseudovorticella sp.2	–	–	–	5	10	–	15	5	–	–	–	–	–	–
Brooklynella sinensis	–	–	–	–	–	5	–	–	–	–	–	–	30	35
Litonotus sp.1	–	–	–	–	–	–	–	–	–	–	10	–	15	20
Protogastrostyla pulchra	–	–	–	–	5	–	5	–	5	–	–	–	–	–
Stephanopogon paramesnili	–	–	–	–	–	–	–	–	10	10	–	20	–	–
Condylostoma sp.	–	–	–	–	–	–	–	–	–	–	5	–	–	10
Leptoamphisiella vermis	–	–	–	–	–	–	–	–	5	–	–	–	–	15
Loxophyllum qiuianumi	–	–	–	–	5	–	–	–	10	–	–	–	–	–
Stichotricha marina	–	–	–	–	–	–	–	–	–	5	–	–	–	10
Chilodonella sp.	–	–	–	–	–	–	10	–	–	–	–	–	–	–
Chlamydodon sp.	–	–	–	–	–	–	–	–	–	–	–	–	5	–
Chlamydonella derouxi	–	–	–	–	–	–	–	–	–	–	–	–	10	–
Diophrys irmgard	–	–	–	–	–	–	20	–	–	–	–	–	–	–
Dysteria cristata	–	–	–	–	–	5	–	–	–	–	–	–	–	–
Holosticha bradburyae	–	–	5	–	–	–	–	–	–	–	–	–	–	–
Litonotus guae	–	–	–	–	–	5	–	–	–	–	–	–	–	–
Loxophyllum jinni	–	–	–	–	–	–	–	–	–	–	–	25	–	–
Loxophyllum simplex	–	–	–	–	5	–	–	–	–	–	–	–	–	–
Pseudotrachelocerca trepida	–	–	–	–	–	–	–	–	–	15	–	–	–	–
Spirotrachelostyla tani	–	–	–	–	–	–	–	–	–	–	–	–	–	5

Species with shaded data, typical species with 99.5% cumulative contributions to each ciliate community.

Table 2. Numbers of slide-replicates required to achieve a 95% probability of recovering species of ciliates with a set of cumulative contributions (90%–100%) to the ciliate communities at depths of 1 m and 3 m at the study site during the study period.

Cum (%)	1-day		3-day		7-day		10-day		14-day		21-day		28-day	
	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m	1 m	3 m
90	18	20	8	7	9	9	4	5	4	3	2	3	4	2
95	28	28	19	14	13	14	6	10	5	4	3	4	6	3
99.5	34	38	31	35	39	42	19	23	15	16	11	14	16	13
100	85	70	95	95	102	99	97	97	88	88	103	88	97	95

Cum = cumulative contribution.

example, the 14-day sample with two slide-replicates has low CV values (<5%) compared with the 1-day sample with eight slide-replicates (<10%) for both species number and abundance (Figures 2c and 3c). It should be noted that eight slide-replicates were sufficient to achieve <10% CV values in species number for the 1-m samples at all colonization periods, but 10 slide-replicates were required for the 3-m samples except for the 3-day colonization period (Figure 2c, d). In terms of abundance, similar trends were found in the samples with colonization periods of >1 day (Figure 3c and d). Furthermore, greater sampling effort (ie more slide-replicates) was needed for young (1–7 day) communities than for the mature (10–21 day) communities in order to achieve the same ciliate species recovery rates regardless of the sampling depths (Figures 2c, d and 3c, d).

Sampling effort required for determining colonization patterns

The colonization patterns of biofilm-dwelling ciliate communities based sampling effort (ie examination of 1–20 replicates) at the two depths were analyzed using the second-stage MDS routine (Figure 4a and b). Cluster analyses showed that there is a high similarity (Spearman rank correlation coefficient $\rho = 0.90$) among the colonization patterns of ciliate communities with 1–20 slide-replicates. The 20 patterns of ciliate colonization can be assigned into four groups comprising the fewest (1, 3, 5 and 10) slide-replicates (Figure 4a and b). However, the colonization patterns showed minor differences between the 1- and 3-m samples. For example, the similarity between the samples with 3–8 slide-replicates and those with 10–20 slide-replicates

was higher at the depth of 1-m depth ($\rho = 0.99$) than at the 3-m depth ($\rho = 0.95$).

The colonization curves of ciliate communities based on the sample sizes of 1, 3, 5, 10 and 20 slide-replicates at the two depths are summarized in Figure 4c and d. Nonparametric test reveals no significant differences among the five colonization curves ($P > 0.05$) in both the 1- and 3-m samples. However, the colonization parameters of ciliate communities with 1, 3, 5, 10 and 20 slide-replicates at both depths during the study period showed a clear increasing (eg S_{eq} and G values) or decreasing ($T_{90\%}$ values) trends with the increasing sample size (Table 3). It should be noted that the colonization rates were comparatively higher at the depth of 1 m than at 3 m. For example, the $T_{90\%}$ values ranged from 12.86 to 20.93 days for the 1-m samples, but 17.71 to 50.06 days for the 3-m samples (Table 3). The analyses show that 10 slide-replicates are sufficient to achieve $<10\%$ CV values in all three colonization parameters (ie S_{eq}, G and $T_{90\%}$) of ciliate communities at both depths (Table 4).

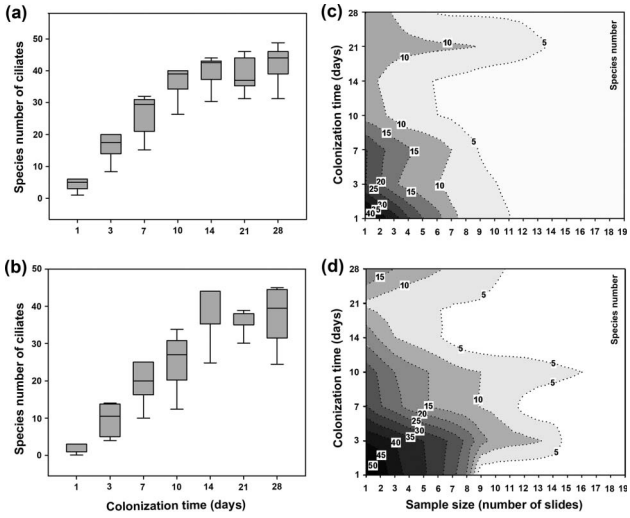

Figure 2. Species numbers of biofilm-dwelling ciliates in 1-, 3-, 7-, 10-, 14-, 21- and 28-day communities based on 1–20 slide-replicates (a, b), and coefficients of variation in species number among the samples based on 1–20, 2–20, 3–20, ... , and 19–20 slide-replicates (c, d), at depths of 1 m (a, c) and 3 m (b, d) at the study site during the study period. Numbers in the panels c and d show the scale-values of the coefficients of variation.

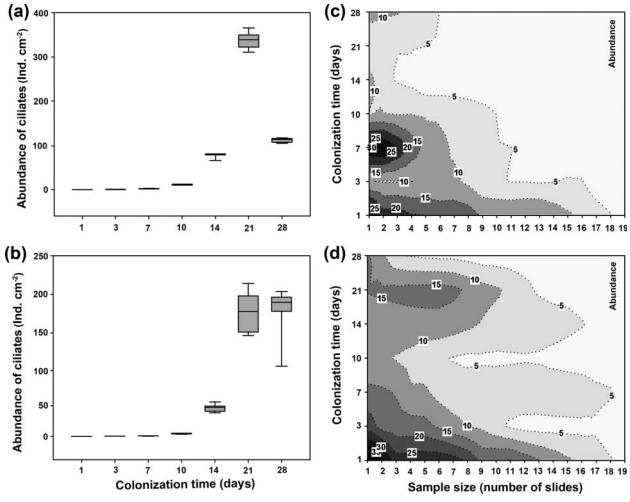

Figure 3. Number of individuals per square cm of biofilm-dwelling ciliates in 1-, 3-, 7-, 10-, 14-, 21- and 28-day communities based on 1–20 slide-replicates (a, b), and coefficients of variation in abundance among the samples based on 1–20, 2–20, 3–20, ... , and 19–20 slide-replicates (c, d), at 1 m (a, c) and 3 m (b, d) depths at the study site during the study period. Numbers in the panels c and d show the scale-values of the coefficients of variation.

Sampling effort for evaluating structural parameters of biofilm-dwelling ciliate communities

Species richness, diversity and evenness of ciliates in 1-, 3-, 7-, 10-, 14-, 21- and 28-day communities based on 1–20 slide-replicates at the two depths during the study period are summarized in Figure 5. The boxplots showed that, at both depths, the variations in all three structural parameters (ie species richness, species diversity and species evenness) increased with decreasing colonization time, and that all three indices showed

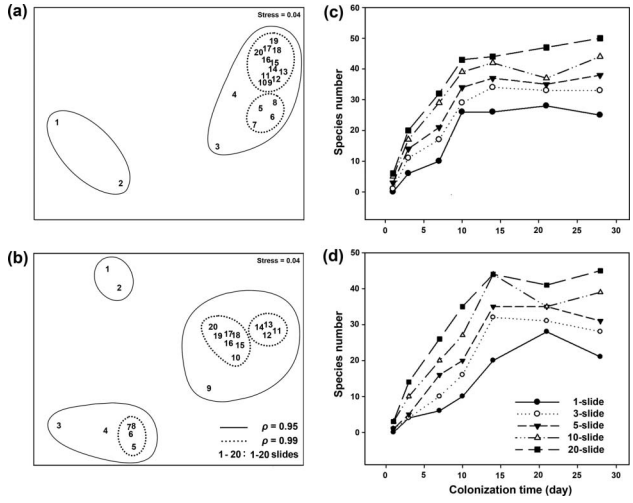

Figure 4. Second-stage MDS on second-stage similarity matrix from the first-stage Bray–Curtis similarity matrices of fourth-root transformed species-abundance data, showing similarities of the colonization patterns of periphytic ciliate communities based on different sampling efforts (1–20 replicates) at depths of 1 m (a) and 3 m (b), and colonization curves of periphytic ciliate communities based on sample sizes of 1, 3, 5, 10 and 20 slide-replicates at the two depths, 1 m (c) and 3 m (d), at the study site during the study period. P = Spearman rank correlation coefficient.

Table 3. Colonization parameters of periphytic ciliate communities based on 1, 3, 5, 10 and 20 slide-replicates at depths of 1 m (a) and 3 m (b) at the study site during the study period.

Parameters	1-slide	3-slide	5-slide	10-slide	20-slide
(a) 1-m samples					
S_{eq}	29.74	36.16	39.16	42.85	49.84
G	0.11	0.13	0.15	0.18	0.16
$T_{90\%}$	20.93	18.27	15.77	12.86	14.13
R^2	0.87	0.94	0.95	0.96	0.99
(b) 3-m samples					
S_{eq}	35.11	37.42	38.65	42.65	46.78
G	0.05	0.07	0.09	0.11	0.13
$T_{90\%}$	50.06	33.06	26.17	20.38	17.71
R^2	0.85	0.87	0.90	0.90	0.97

S_{eq}, the estimated equilibrium species number in ciliate colonization; G, the colonization rate constant; $T_{90\%}$, the time taken to reach 90% S_{eq}; R^2, regression coefficient.

Table 4. Coefficients of variation in colonization parameters of periphytic ciliate communities at depths of 1 m (a) and 3 m (b) at the study site during the study period.

Parameters	1-slide	3-slide	5-slide	10-slide
(a) 1-m samples				
S_{eq}	18.95	14.04	12.34	9.66
G	19.12	14.82	10.14	6.62
$T_{90\%}$	19.79	15.30	10.24	6.65
(b) 3-m samples				
S_{eq}	37.63	26.92	19.15	9.89
G	11.51	10.25	9.52	6.53
$T_{90\%}$	43.85	28.44	20.19	9.91

S_{eq}, the estimated equilibrium species number in ciliate colonization; G, the colonization rate constant; $T_{90\%}$, the time taken to reach 90% S_{eq}.

higher values in the 1-m than the 3-m samples (Figure 5). It should be noted that all three indices in 28-day samples at the depth of 1 m sharply increased to a high level, mainly due to the decrease of abundance. Furthermore, the contour plotting analyses showed that: (1) 8 and 14 slide-replicates were sufficient to achieve $<10\%$ CV values in species richness (Figure 6a and b), and (2) for 1- and 3-m samples, 5 and 9 slide-replicates respectively were the minimum required to achieve $<10\%$ CV values in species diversity and evenness (Figure 6c–f).

Discussion

Based on the results, the required number of slide-replicates will vary according to both the occurrences and contributions of ciliate species to each community within different colonization periods, and thus an optimal sample size is difficult to define. For achieving an assemblage of ciliates with higher cumulative

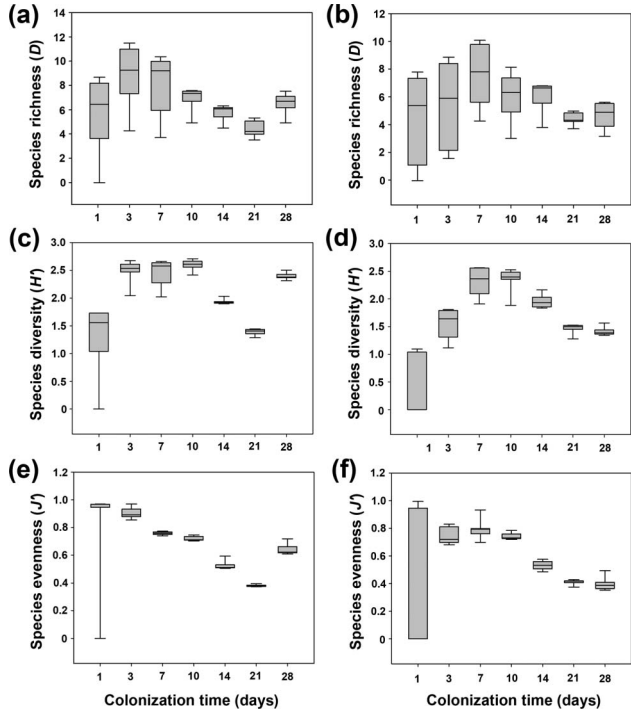

Figure 5. Species richness (a, b), diversity (c, d) and evenness (e, f) of biofilm-dwelling ciliates in 1-, 3-, 7-, 10-, 14-, 21- and 28-day communities based on 1–20 slide-replicates at depths of 1 m (a, c, e) and 3 m (b, d, f) at the study site during the study period.

contribution to communities using an optimum sampling effort, it is necessary to neglect the rare species. The data suggest more slide-replicates are needed within shorter colonization times. To recover the ciliate species expected with a cumulative contribution of 95% at depths of 1 m and 3 m, for example, 3–10 slide-replicates are sufficient within colonization times more than 7 days, while 14–19 slides were needed within colonization times of 3–7 days. These findings suggest that 10 and 20 slide-replicates are sufficient to recover the species with cumulative contributions of at least 90% and 95% to the ciliate communities within the exposure periods longer than 1 day.

The ciliate communities with colonization times 10 days or more are generally considered sufficiently mature for the evaluation of temporal/spatial variations in response to the changes of environmental conditions (Coppellotti and Matarazzo 2000; Norf et al. 2009b; Xu et al. 2009b). Based on the present data, 5 and 10 slide-replicates resulted in a probability of 95% of recovering all species expected to achieve a cumulative contribution of $> 95\%$ to the mature communities of the ciliates at the depths of 1 m and 3 m, respectively. Thus, fewer than five slide-replicates, which are commonly used as the sampling effort strategy in some previous investigations, might

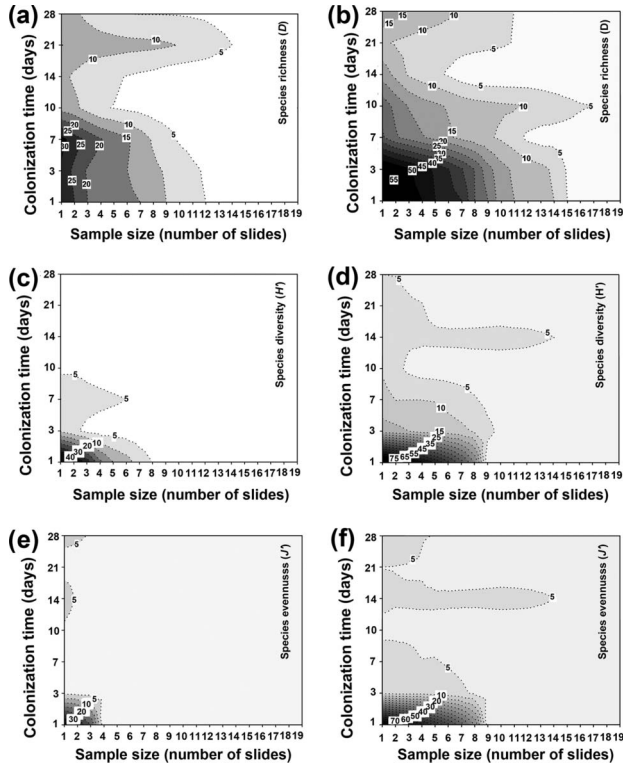

Figure 6. Coefficients of variation in species richness (a, b), diversity (c, d) and evenness (e, f) of biofilm-dwelling ciliate communities at depths of 1 m (a, c, e) and 3 m (b, d, f) at the study site during the study period, among samples based on 1–20, 2–20, 3–20, . . . , and 19–20 slide-replicates. Numbers in the panels show the scale-values of the coefficients of variation.

achieve >95% probabilities of recovering the species with a cumulative contribution of 90% (Coppellotti and Matarazzo 2000; Gong et al. 2005). However, analyzing just two or three slides for each sample in some previous studies on periphytic ciliate communities process would result in <95% probabilities of recovering all species expected (Coppellotti and Matarazzo 2000; Xu et al. 2009a).

In the present study, the analyses of CV values showed that the standard errors were strongly dependent on the sampling effort in terms of both species number and abundance, eg the greater the number of slide-replicates, the lower the CV values. Based on the data, eight slide-replicate were sufficient to achieve <10% CV values in species number and abundance for all colonization periods for the 1-m samples, but for the 3-m communities 10 slide-replicates were required (apart from the 1-m and 3-day samples).

Structural parameters, such as species richness, species diversity and species evenness, are commonly used to summarize the response of a community to environmental change (Ismael and Dorgham 2003; Jiang et al. 2007; Xu et al. 2009b; Tan et al. 2010). However, these indices present a number of problems for quantifying environmental changes due to their dependence on sample size or sampling-effort (Warwick and Clarke 2001; Leonard et al. 2006; Prato et al. 2009). Based on the present results, in order to achieve <10% coefficients of variation in all three parameters of ciliate communities within different colonization periods, ~10 slide-replicates are sufficient, or only one or two slide-replicates for the mature (eg 14-day) ciliate communities at depths of both 1 m and 3 m. However, it should be noted that the sampling effort needed for evaluating these biotic parameters is greater at a depth of 1 m than at 3 m.

Colonization dynamics and functional parameters are usually used to predict the loading capacity or assimilative capacity of an aquatic ecosystem for contaminant inputs (Xu et al. 2002). Among the functional parameters, the S_{eq} value (estimated equilibrium species number) is generally negatively correlated with concentrations of organic pollutants and toxic levels, while the G value (colonization rate constant) is generally high in waters with lower environmental stress (Cairns and Henebry 1982; Xu et al. 2002, 2009a). Based on the present data, all three parameters present strong sampling-effort dependence. In contrast, the various colonization patterns showed high similarities regardless of the sampling effort. In order to achieve <10% coefficients of variation in functional parameters, 10 slide-replicates were sufficient for the ciliate communities at both depths in the coastal waters of the Yellow Sea.

It should also be noted that both the abundance and occurrence of biofilm-dwelling ciliates were lower at a depth of 3 m than at 1 m. This was probably due to weaker sunlight levels and lower food availability at the deeper level in the water columns. This may explain why fewer slide-replicates were needed for evaluating both community patterns and colonization dynamics of biofilm-dwelling ciliates at a depth of 1 m compared to 3 m. Additionally, the abundances in 28-day samples collected at 1 m dropped sharply to a low level, mainly due to the intensive immigration of metazoan consumers (eg copepods, coelenterates, annelids and barnacles). This implies that the ciliate community structure changed significantly after the 28-day exposure time.

In summary, the optimal sample size for evaluating biofilm-dwelling ciliate colonization increases with shortening colonization time, and can be determined according to a probability of recovering the species with a specified cumulative contribution to communities. More slide-replicates were required at a depth of 3 m than at 1 m in order to recover equivalent proportions of the ciliate communities. For routine

analyses of colonization dynamics at a depth of 1 m, 10 slide-replicates (total area 175 cm^2) were sufficient to achieve a 95% probability of recovering the species with a cumulative contribution of $>90\%$ to the ciliate communities. Thus, it is suggested that a 10 slide-replicate sample size with a sampling period of <28 days and a depth of 1 m is the optimal sampling strategy for analyzing biofilm-dwelling ciliate colonization in the coastal waters of the Yellow Sea. Further studies, however, on a range of marine waters and over extended time periods are needed in order to verify this conclusion.

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