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Detection of the bloom-forming cold-water dinoflagellate *Biecheleria baltica* in the Baltic Sea using LSU rRNA probes

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ABSTRACT: Cold-water dinoflagellates frequently dominate the spring phytoplankton community of the northern Baltic Sea and contribute substantially to the spring primary production. These dinoflagellate communities are largely composed of 3 different species (*Biecheleria baltica*, *Scrippsiella hangoei*, *Gymnodinium corollarium*) that cannot be unambiguously separated by conventional light microscopy. In this study, a fluorescence *in situ* hybridization (FISH) assay was developed for the detection of *B. baltica* in field samples. The probe-based method was tested and optimized *in vitro*, and potential effects of the *B. baltica* life-cycle transitions were evaluated by examining the labeling quality of the probes on different life-cycle stages before the assay was applied in a field study. The BbRNA4 probe had the highest specificity and was chosen for downstream applications. The life-cycle experiment showed significant differences in labeling efficiency between cultured cells from different growth phases, particularly for the nutrient-limited treatment compared to the control. Tests with spiked field samples revealed that cell recovery in the FISH assay was low (30%), resulting in a considerable underestimation of *in situ* abundances. However, a strong relationship between FISH and Utermöhl counts of field samples suggests that trends of the bloom dynamics can be followed reliably by this method. By applying the FISH detection method in a field survey, we found that the dynamics of the *B. baltica* bloom follows that established earlier for the *Scrippsiella/Biecheleria/Gymnodinium* complex, with 2 peaks throughout the season. When corrected for processing losses, FISH-based abundance estimates suggest that *B. baltica* comprised the major fraction of the total dinoflagellate complex during the spring bloom at the southwest coast of Finland.

KEY WORDS: Baltic Sea · Dinoflagellates · FISH · Gulf of Finland · LSU rRNA · Molecular detection · Probes · Spring bloom

INTRODUCTION

Cold-water dinoflagellates are common in the spring phytoplankton community of the northern Baltic Sea. They frequently dominate the spring bloom and constitute a major fraction of the newly produced biomass (Lignell et al. 1993, Heiskanen & Kononen 1994, Larsen et al. 1995). The taxonomic affiliations of the involved species have long been unclear. Recent morphological and molecular analyses on cultured isolates revealed that at least 3 different species, which are inseparable by light microscopy, may be associated

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with these blooms (Larsen et al. 1995, Kremp et al. 2005, Sundström et al. 2009). It was established that *Biecheleria baltica* Moestrup, Lindberg et Daugbjerg (= *Woloszynskia halophila* sensu Kremp et al. 2005) (Moestrup et al. 2009), co-occurs with *Scrippsiella hangoei* (Schiller) Larsen at the southwest coast of Finland (Kremp et al. 2005), whereas a third recently described species, *Gymnodinium corollarium* A. M. Sundström, Kremp et Daugbjerg (Sundström et al. 2009), possibly occurs throughout the Baltic Sea.

Due to their similar size and shape, the vegetative cells of the 3 species cannot be unambiguously distinguished from one another using light microscopy, particularly in samples preserved with Lugol's iodine solution (Kremp et al. 2005, Sundström et al. 2009). Only *Scrippsiella hangoei* possesses a theca with a specific plate pattern that could aid light microscopic identification. However, the plates of this species are unusually delicate and may be only weakly visible (or not visible at all) even when stained with fluorescent brightener (Larsen et al. 1995). In both *Biecheleria baltica* and *Gymnodinium corollarium*, the specific cell surface structures can only be detected by scanning electron microscopy analyses, which involve elaborate procedures and are hardly applicable for field surveys and routine monitoring. All 3 species produce distinctive resting cysts, which may provide some information about their presence in the spring bloom. However, the information on distribution and abundances that can be derived from cyst data is limited, as the different species may have different encystment strategies. The part of the population that undergoes encystment may vary between and within species depending on prevailing environmental conditions (Kremp et al. 2009). To reliably assign the vegetative cells to the respective taxon and to investigate the distribution, environmental requirements, and population dynamics of the separate species, an identification method is needed that is not based solely on morphological features.

Among the tools for dinoflagellate species identification, ribosomal gene sequence (rDNA) comparisons between species can be used to discriminate between species with similar morphology (Anderson 1995). Fluorescence *in situ* hybridization (FISH) coupled with ribosomal RNA (rRNA) probes is one of the most popular molecular methods utilized to detect dinoflagellates and has been successfully applied in the detection and quantification of several species (Litaker & Tester 2002, John et al. 2003, 2005, Touzet & Raine 2007). Many studies of harmful algal bloom species have applied FISH analyses (e.g. Goodwin et al. 2005, Hosoi-Tanabe & Sako 2005, Kim et al. 2005, Touzet et al. 2009). Among others, Mikulski et al. (2005) and Touzet et al. (2009) successfully applied rRNA probes to cultures and field samples. The FISH technique identifies cells of different species by coupling a species-specific rDNA sequence with a fluorochrome which penetrates the cell and binds to the rRNA. Hence, the numerous ribosomes in cells harvested from the exponential growth phase constitute a natural signal amplification system (Pernthaler et al. 2001). Specificity of probe binding to the target site is mainly dependent on the hybridization and washing conditions. Several factors affect the melting behavior of the rRNA probes, including temperature, composition of the hybridization buffer, and the length and sequence of the probe (Pernthaler et al. 2001). During the optimization process, the probe is hybridized with both target and non-target species, and the conditions for maximum probe specificity are established. An advantage with whole cell hybridization is that the features of cell morphology stay intact, permitting their visualization under the epifluorescence microscope (Groben et al. 2004). FISH is a sensitive detection method, although the number of detected cells depends on sample volume, as high cell densities can obscure the view of target cells (Karlson et al. 2010).

In this study, an rRNA-targeted probe was developed for *Biecheleria baltica,* which allows this species to be distinguished from co-occurring dinoflagellates in the natural phytoplankton community. According to cyst data, the species seems to be particularly abundant in the spring dinoflagellate community at the Finnish coast. As a large fraction of the *B. baltica* population usually encysts (Kremp & Heiskanen 1999), potential biases in abundance estimates due to life-cycle transitions were evaluated by examining the labeling quality of the probes in different life-cycle phases. Finally, to demonstrate the utility of the method, the selected probes were applied to natural samples from the southwest coast of Finland collected throughout a spring bloom.

MATERIALS AND METHODS

Cultures. The rRNA probes were tested on unialgal cultures of *Biecheleria baltica, Scrippsiella hangoei*, *Gymnodinium corollarium*, and 2 other dinoflagellate species that co-occur in the Baltic Sea during spring, *Heterocapsa triquetra* and *Peridiniella catenata*. The cultures were maintained in temperature regulated incubators at 50 µmol photons m^{-2} s^{-1} and a 14:10 h light:dark cycle. F/2-Si enrichments (Guillard & Ryther 1962) with salinity of 6.5 were used for growth media preparations for all cultures (salinities are given in practical salinity units [psu] throughout this paper). All cultures were maintained at 4°C, except *H. triquetra*, which was grown at 17°C. Strains used in this study and their origin are summarized in Table 1.

Table 1. Dinoflagellate strains used in this study and their origin

Species	Strain code	Origin
Biecheleria baltica	WHTV-10	Tvärminne (SW coast of Finland)
B. baltica	WHTV-11	Tvärminne
B. baltica	WHTV-C1	Tvärminne
B. baltica	WHTV-C6	Tvärminne
B. baltica	WHTV-S1	N Baltic proper, Sweden
B. baltica	WHTV-S2	N Baltic proper, Sweden
B. baltica	WHTV-S3	N Baltic proper, Sweden
Scrippsiella hangoei	SHTV-1	Tvärminne
S. hangoei	SHTV-2	Tvärminne
S. hangoei	SHTV-5	Tvärminne
Gymnodinium corollarium	GCTV-B4	N Baltic proper, Sweden
G. corollarium	GCTV-C5	N Baltic proper, Sweden
Peridiniella catenata	PCTV-1	Tvärminne
Heterocapsa triquetra	K-0447	Nivå Bugt, The Danish Sounds, Denmark

Table 2. Species, available strains, and GenBank accession numbers included in the LSU rDNA sequence alignment

Species	Strain	Accession no.
Alexandrium tamarense	K-0055	AF200668
Amphidinium carterae	JL3	AF260380
Biecheleria baltica	WHTV-C1	AY628430
Cochlodinium polykrikoides		AF067861
Gymnodinium aureolum	K-0303	AF200671
G. catenatum	GCCC16	AY036071
G. chloroforum	K-0539	AF200669
G. fuscum	CCMP1677	AF200676
G. galatheanum	K-0522	AF200675
G. microreticulatum	GMNC01	AY036078
G. mikimotoi	K-0579	AF200682
Gyrodinium dominans		AY571370
G. spirale		AY571371
Heterocapsa rotundata	K-0479	AF260400
H. triquetra	K-0447	AF260401
Peridiniella catenata	K-0543	AF260398
Peridinium palatinum	AJC4cl-a	AF260394
P. willei	AJC2-675	AF260384
Protodinium simplex		AY686651
Scrippsiella hangoei	SHTV1	AY628426
S. trochoidea	GeoB*238	AY628427
Woloszynskia tenuissima		AY571374

Sequence alignment and probe design. Probes for the variable D1/D2 region of the large subunit (LSU) were designed by aligning the LSU rDNA sequence of *Biecheleria baltica* generated by Kremp et al. (2005) with the sequences of the species shown in Table 2, using ClustalX (version 1.83) with default settings and manual checking of the alignment. Fragments considered for probe design were chosen to exhibit at least 1 mismatch between *B. baltica* and non-target species. Wherever possible, the probes were designed to bind to the looped domains of the RNA molecule in order to improve the binding of a probe during hybridization. For the sequence of *B. baltica*, the locations of the looped domains in its secondary structure were predicted by modeling using mfold version 3.1.2a (online web server, Mathews et al. 1999). The rRNA probes were manually designed to contain 18 to 26 base pairs. A database similarity search was made using BLAST to determine if the probes could bind to sequences in other species. The probes tagged with a fluorochrome (fluorescein) at the 5′ end (Oligomer) were used at a 0.2 µg μl^{-1} final concentration. The sequences of the tested probes are given in Table 3. The positive control probe (UniC) is designed to target a universally conserved sequence of the small subunit, and the negative control probe (UniR) is the reverse complement of UniC (Miller & Scholin 2000).

Probe testing. The specificity and cross-reactivity of the generated rRNA probes were evaluated in labeling experiments using unialgal cultures harvested during exponential growth. Initial assessment of species-specific binding of the probes was performed on the *Biecheleria baltica* isolate WHTV-10, after which the probe testing was extended to the other *B. baltica* strains. The probes that bound successfully to all *B. baltica* strains were then used in cross-reactivity tests with potentially co-occurring dinoflagellate species.

Table 3. Sequences and properties of the tested rRNA probes. Tm: melting temperature (variable if ambiguities occur); +: positive hybridization, –: negative hybridization

Name	Target species	Probe sequence (5′–3′)	Tm (°C)	Length (bp)	Binding to *B. baltica*	Probe source
BbRNA2	*Biecheleria baltica*	GAA ACA CCA CAC ACA AAA GC	55.3	20	+	This study
BbRNA3	*B. baltica*	CTT TAC GCT CCA ATT CCG AGG AAC GC	66.4	26	+	This study
BbRNA4	*B. baltica*	AAC ACA GGA TTC TCA CCC ACG	59.8	21	+	This study
BbRNA5	*B. baltica*	GGT GCT ATG TCT AGA GGC	56.0	18	+	This study
BbRNA6	*B. baltica*	AAG CAC AGG AGC AAC CCC	58.2	18	+	This study
UniC	Positive control	GWA TTA CCG CGG CKG CTG	59.4	18	+	Miller & Scholin (2000)
UniR	Negative control	CAG CMG CCG CGG XAA XWC	59.4	18	–	Miller & Scholin (2000)

To enhance specificity, different concentrations of formamide were applied to the hybridization buffer, beginning at 10% and increasing by 5% to a maximum concentration of 35%. The stringency of the tested probes was further increased by increasing the hybridization temperature from 50 to 54°C and finally to 58°C.

Sample fixation. The fixation protocol outlined below was adapted from Mikulski et al. (2005). A 20 ml aliquot of culture was gently mixed with 20 ml of modified saline ethanol solution (MSES; volume ratios according to Miller & Scholin 2000: 22 ml 95% ethanol, 5 ml deionized H_2O ,and 3 ml 25× SET buffer — 3.75 M NaCl, 25 mM EDTA, 0.5M Tris pH 7.8, all in deionized H_2O, sterile filtered through a 0.45 µm filter unit). The mixture was allowed to fix for 1 h at room temperature, whereupon 4.4 ml formalin (37% formaldehyde) were added followed by additional fixation for 10 min at room temperature. The preserved samples were stored in the dark at 4ºC until processing.

Hybridization. White polycarbonate membrane filters (25 mm, pore size 3.0 µm, Nucleopore, Whatman) were placed in a filter unit fitted with chimneys (Carbon 14 Centralen). The complete filter unit was connected to a vacuum pump (Millipore, Model XX5522050). An aliquot of fixed sample corresponding to sufficient material of approximately 5000 cells (determined with a Sedgewick-Rafter cell; McAlice 1971) was added to each filter and filtered to near dryness. One ml of pre-hybridization buffer — 250 µl formamide, 200 µl 25× SET, 10 µl 10% IGEPAL, 10 µl Poly A (10 mg ml^{-1}) in 530 µl deionized H_2O — was added and the cells were pre-hybridized for 15 min at room temperature. After filtration, 990 µl of hybridization buffer (probe diluted in pre-hybridization buffer; final probe concentration 4.7 ng $µl^{-1}$) were added. The chimneys were capped and the filter unit was covered with a black plastic bag containing a wet paper towel to minimize evaporation. The filter unit was placed in an incubator (Thermo Scientific Heraeus, model B6, Thermo Fisher Scientific) set to 58°C, and probes were allowed to hybridize with the cells for 2 h. After incubation and filtration of the hybridization buffer, 1 ml of preheated (60°C) 0.1× SET washing buffer was added and the filter unit was incubated for 10 min at room temperature. The samples were filtered and mounted on slides using 25 µl of Slowfade Light anti-fade solution (Molecular Probes). The prepared slides were viewed immediately under the epifluorescence microscope.

Epifluorescence microscopy. The assessment of probe binding was performed manually in an epifluorescence microscope (Leica DM IRB, Leica Microsystems) at 250× magnification (50 W lamp, fluorescein isothiocyanate selective L5 filter, excitation wavelength 460 to 500 nm). The whole filter was counted. Epifluorescence images were taken with a Leica DC300F camera and processed using Leica IM 50 Image Manager software.

Life-cycle experiment. To evaluate the probe binding efficiency on different phases of the *Biecheleria baltica* life cycle, cyst formation was induced in a mixed culture of *B. baltica* strains WHTV-C6, WHTV-11, and WHTV-S1. As encystment of dinoflagellates in culture has been successfully induced by nutrient limitation (Figueroa & Bravo 2005), reduced phosphorus concentrations (–P, f/20 phosphorus concentrations) in the growth media were used to trigger cyst formation. Nutrient-replete conditions served as a control treatment (control, f/2 enrichments). Exponentially growing cultures were inoculated into multiple 50 ml culture vessels containing 40 ml of the respective medium, at an initial cell concentration of 800 cells ml^{-1}. The flasks were incubated for 4 wk at 4°C, irradiance 50 µmol photons m^{-2} s^{-1}, and a 14:10 h light:dark cycle.

Samples were taken once in early exponential growth phase (Day 7) and once in stationary phase (Day 29). Five replicate flasks were randomly chosen from each treatment group, and for every flask a 1.3 ml subsample was fixed with Lugol's solution for light microscopic cell and cyst counts. Motile cells were grouped into 2 different size categories, where cells >27 µm were assumed to be planozygotes, the motile sexual stage preceding the actual encystment (A. Kremp pers. obs.). To examine FISH labeling quality, 27.5 ml of the remaining sample were collected. Fixation of samples in this experiment followed an alternative fixation protocol, F/M fixation, based on formalin and methanol (adapted from Godhe et al. 2002), that had been successfully tested on spiked sea water samples. Sample volumes for hybridization were adjusted so that at least 5000 vegetative cells were retained on each filter, using cell numbers obtained by the cell counts. The cells were hybridized as described above, and the proportion of labeled cells to total vegetative cells on the filter was determined by epifluorescence microscopy. Statistical data analyses (*t*-tests) were performed in the Microsoft Office Excel software.

Assessment of FISH detection efficiency in cultures and natural sea water. To evaluate detection efficiency and sensitivity of the FISH assay, FISH counts were compared to conventional cell counts, using 1 mixed and 3 clonal cultures of *Biecheleria baltica* (WHTV-C1, WHTV-11, and WHTV-S1). A sample of the culture was divided into 2 subsamples, of which 1 was enumerated by light microscopy in a Sedgewick-Rafter chamber (1 ml) after fixation with acid Lugol's solution. The other sample was hybridized and counted using epifluorescence microscopy as described above.

Furthermore, a spiking experiment was performed to test probe binding, cell recovery, and possible matrix effects in a natural water sample with a phytoplankton community containing only *Biecheleria baltica* of the *Biecheleria/Scrippsiella/Gymnodinium* complex. Natural sea water was collected at Tvärminne Zoological Station, off the southwest coast of Finland, in late summer when — due to the high water temperature — the complex is not present in the plankton (Kremp & Anderson 2000). Three carboys (8 l) of seawater (4°C) were inoculated with *B. baltica* strains WHTV-S1 and WHTV-11 at a range (5000 to 500 000 cells l^{-1}) similar to ambient spring concentrations expected for the complex. A fourth sample without *B. baltica* addition served as a negative control. The contents of the carboys were mixed carefully before samples were taken and processed immediately for FISH labeling (as described below for field samples) and conventional cell counts using the Utermöhl method (Utermöhl 1958). For the Utermöhl counts, the samples were conserved with Lugol's iodide solution (0.4 ml per 100 ml sample), carefully mixed and settled overnight in 10 ml (detection limit 200 cells l^{-1}) or 50 ml (detection limit 40 cells l^{-1}) chambers, depending on cell density, after which half the bottom plate was surveyed.

Application of the FISH assay in the field. The FISH detection assay developed for *Biecheleria baltica* was applied in a field survey, monitoring the bloom development of the species at the southwest coast of Finland. Samples were collected in the vicinity of the Tvärminne Zoological Station from inshore station Storfjärden (59° 51′ N, 23° 15′ E, depth 35 m) and offshore station Längden (59° 46′ N, 23° 16′ E, depth 78 m) during spring 2005, in approximately 2 wk intervals from late February to late May. Water samples collected from 1, 2.5, 5, 7.5, 10, 12.5, and 15 m depths, with a 2 l Ruttner water sampler, were pooled, stored at 4°C in the dark, and processed within 24 h. A subsample of 300 ml was preserved with acid Lugol's solution, and cell numbers of the *Biecheleria/Scrippsiella/Gymnodinium* complex were determined according to the Utermöhl method. Hydrochemical and hydrographic data (dissolved inorganic phosphorus, DIP, dissolved inorganic nitrogen, DIN, salinity, and temperature) were provided by the Tvärminne Zoological Station monitoring program for Storfjärden and by the Uusimaa Regional Environment Centre for Längden.

For hybridization, 5 l of the sample water were concentrated. The water was pre-screened with a 45 µm sieve to remove large phyto- and zooplankton and then concentrated on a 10 µm sieve. The retained material was collected into a 50 ml centrifuge tube and fixed in the same manner as outlined for cultures above (MSES fixation). Using cell numbers for the *Biecheleria/Scrippsiella/Gymnodinium* complex obtained by the Utermöhl method, sample volumes were adjusted so that at least 5000 cells of the complex were retained on each filter. The hybridization and epifluorescence microscopy of field samples were carried out as described for the cultures. Statistical data analyses (regression and inspection of residuals) were performed using the Statistica software package (StatSoft).

RESULTS

Probe specificity

A bright green fluorescence signal throughout the cytoplasm, often with a ring-like pattern around the nucleus, showed successful binding of probes to the rRNA of the tested phytoplankton (Fig. 1). Five generated rRNA probes showed positive labeling to all *Biecheleria baltica* strains (Table 3). The probes BbRNA3 and BbRNA4 exhibited the brightest fluorescent signal and were chosen for cross-reactivity testing. In parallel application of the probes on other species, BbRNA4 was more specific than BbRNA3 and was therefore used in further validation studies. In the initial trials, where the concentration of formamide in the hybridization buffer was 10% and the hybridization temperature was 50°C, BbRNA4 hybridized non-specifically with *Heterocapsa triquetra* and *Scrippsiella hangoei*. Binding specificity improved by increasing temperature and formamide concentration, and eliminated the cross-reactivity of BbRNA4 for these 2 species. Further increases of formamide concentration (30, 33, and 35%) yielded a diffuse appearance of the cells under the epifluorescence microscope; hence, a concentration of 25% was chosen as a compromise between stringency and effects on visual appearance. The binding properties at every formamide concentration were consistent for all tested strains of the respective species (Table 4). Non-specific binding of the probe to *Peridiniella catenata* and *Gymnodinium corollarium* was never observed. The positive control probe reacted with all species tested, whereas the negative control probe did not, indicating adequate hybridization conditions (Fig. 1). Positive hybridization of cysts was never observed.

When tested with BLAST, the BbRNA4 probe showed a 100% match with several species, such as *Biecheleria pseudopalustris* (Schiller) Moestrup, Lindberg et Daugbjerg, *Polarella glacialis* Montresor, Procaccini et Stoecker, *Protodinium simplex* Lohmann, *Gymnodinium corii* Schiller, and *G. beii* Spero.

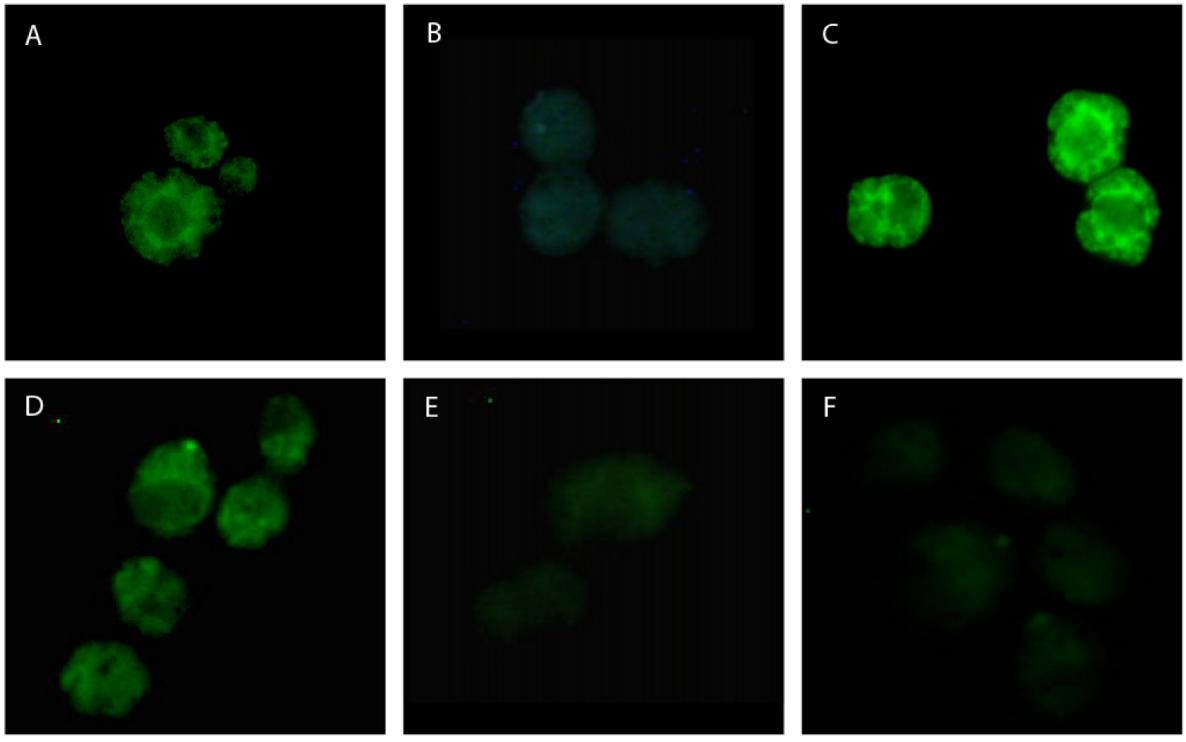

Fig. 1. Epifluorescent images of cultured cells after fluorescence *in situ* hybridization. *Scrippsiella hangoei* is included to show lack of cross-reactivity. (A) Positive control probe with *Biecheleria baltica* WHTV-10 (positive binding). (B) Negative control probe with *B. baltica* WHTV-10 (negative binding). (C) Probe BbRNA4 (*B. baltica* specific) with *B. baltica* WHTV-10 (positive binding). (D) Positive control probe with *S. hangoei* SHTV-2 (positive binding). (E) Negative control probe with *S. hangoei* SHTV-1 (negative binding). (F) Probe BbRNA4 (*B. baltica* specific) with *S. hangoei* SHTV-1 (negative binding)

Table 4. Cross-reactivity of the BbRNA4 probe at different formamide concentrations. +: positive hybridization, –: negative hybridization, +/–: unclear hybridization, empty cell: no data

Species	Strain	Formamide concentration (%)					
		10	20	25	30	33	35
Gymnodinium corollarium	GCTV-B4			–	–		
	GCTV-C5			–	–		
Heterocapsa triquetra	K-0447	+/–	–	–			–
Peridiniella catenata	PCTV-1			–	–	–	–
Scrippsiella hangoei	SHTV-1	+/–	–	–	+/–	–	–
	SHTV-2	+/–	–	–	+/–	–	–
	SHTV-5	+/–	–	–	+/–	–	–
Biecheleria baltica	WHTV-10	+	+	+	+	+	+
	WHTV-11	+	+	+	+	+	+
	WHTV-C1	+	+	+	+	+	+
	WHTV-C6	+	+	+	+	+	+
	WHTV-S1	+	+	+	+	+	+
	WHTV-S2	+	+	+	+	+	+
	WHTV-S3	+	+	+	+	+	+

Life-cycle experiment

The presence of cysts and large cells on Day 29 of the life-cycle experiment, particularly in the low P treatment (Table 5), indicates that the transition from vegetative growth to a sexual resting stage was in progress. In the low P treatment, cyst numbers amounted to approximately 47% of the cell population, compared to Day 7, when nearly 100% of the cells were <27 µm exponentially growing cells. On Day 29, the >27 µm cells presumed to be planozygotes contributed 9 and 10% in the control and low P treatments, respectively. At this point, the contribution of both cysts and large cells was significantly higher compared to Day 7 in both the control (*t*-test: $p = 0.003$ and <0.001, respectively) and the low P treatment (*t*-test: $p = 0.005$ and 0.001, respectively).

While in both treatments nearly 100% of the cells on the filter labeled with the FISH probe at the beginning of the experiment (98 and 99% for the control and low P treatment, respectively), the percentage of FISH labeled cells had slightly decreased on Day 29 in both treatments (*t*-test: control, $p = 0.006$; low P, $p < 0.001$). At the end of the experiment, 96% of the motile cells hybridized in the control treatment,

Table 5. Concentrations (ml^{-1}) of different cell types representing different life cycle stages in the encystment experiment (mean ± SD, n = 5). Two sampling occasions and 2 different treatments are displayed. F/20 P: reduced phosphorus concentration

	Cell type	Control	F/20 P
Day 7	<27 µm	1698 (± 362)	2275 (± 629)
	>27 µm	0	90 (± 49)
	Cysts	0	5 (± 11)
Day 29	<27 µm	3955 (± 973)	770 (± 265)
	>27 µm	445 (± 156)	180 (± 67)
	Cysts	800 (± 362)	800 (± 146)

whereas this portion for the low P treatment had decreased to 92% (*t*-test: p = 0.001).

Labeling efficiency in cultures and natural sea water samples

Comparison of conventional cell counts and cell concentrations obtained by the FISH assay revealed a high frequency of labeling in all tested cultures. We found that 68 to 87% (mean = 80%, SD = 10%, n = 4) of the cells were retained on the filter and showed the fluorescent signal of the probe. In natural sea water (not containing the species complex due to summer water temperature) spiked with *Biecheleria baltica* cells, cell concentrations obtained by the FISH assay were 24 to 39% (mean = 30%, SD = 8%) of the concentrations determined by the Utermöhl method. No evident cross-reactivity with other species was observed when analyzing the spiked field samples. We also did not detect any substantial physical matrix effects, although some autofluorescence of non-hybridized species occurred. However, the autofluorescent cells could clearly be distinguished from hybridized cells due to the different staining pattern of the probe. The negative control sample did not hybridize with the *B. baltica* specific probe, and no *B. baltica* cells were found by the Utermöhl method. The positive control probe (UniC) hybridized with all samples, whereas the negative control probe (UniR) did not.

Spring bloom field study

The BbRNA4 probe hybridized positively with all field samples, and no apparent cross-reactivity with other spring bloom species was observed (Fig. 2). The fluorescent signal of the BbRNA4 probe was very clear until late April, and the target cells were easy to distinguish from the non-target cells. In the May samples, the fluorescent signal was slightly weaker, and autofluorescing cysts that occurred at high numbers in the last samples complicated the cell counts of the samples from Storfjärden on 6 May and Längden on 10 May.

Cell concentrations of *Biecheleria baltica* determined by the FISH assay followed a similar pattern of succession at both Storfjärden and Längden (Fig. 3A). The cell densities were generally higher at the offshore station Längden than inshore at Storfjärden. At the end of March, shortly before the break-up of the ice cover, *B. baltica* reached a first abundance peak at both stations. A second peak, again with considerably higher abundances (a factor of 2) at offshore Längden, occurred approximately 1 mo later. Based on regression analyses, the *B. baltica* abundance determined for the second peak was underestimated (negative outlier). In mid- to late May, the *B. baltica* bloom began to decline at both stations following the decline of dissolved nutrient concentrations in the surface layer (Fig. 3B) and the concurrent rise in water temperature (Fig. 3C). The salinity was 5.1 to 6.0 at Storfjärden and 5.5 to 5.8 at Längden throughout the sampling period.

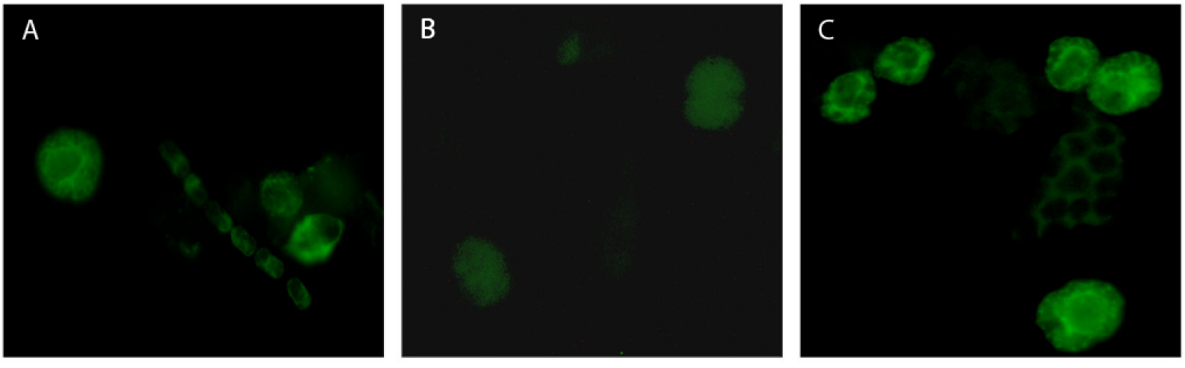

Fig. 2. Epifluorescent images of field samples after fluorescence *in situ* hybridization. (A) Positive control probe hybridized with a sample from Storfjärden showing positive labeling of all cells. (B) Negative control probe hybridized with a sample from Storfjärden showing no labeling. (C) Probe BbRNA4 (*Biecheleria baltica* specific) hybridized with a sample from Storfjärden showing positive labeling of *B. baltica* cells

The abundances determined by the FISH assay were considerably lower than the concentrations obtained by the Utermöhl method. Despite this discrepancy, the same seasonal pattern was reflected by both methods as emphasized by strong regressions obtained for both stations (Fig. 4, Storfjärden $R^2 = 0.95$, $p < 0.001$; Längden $R^2 = 0.99$, $p < 0.001$). Moreover, the slopes of the regression lines are not statistically different (Statistica, general linear model) and indicate a relatively constant percentage (21 to 22%) of *Biecheleria baltica* in the species complex. The 2 data points derived from the complicated cell counts in late spring were identified as outliers in the regression analysis by inspection of residuals. Investigation of the effects of deleting different high values on the regression parameters also supported treating the 2 data points as outliers (Appendix 1).

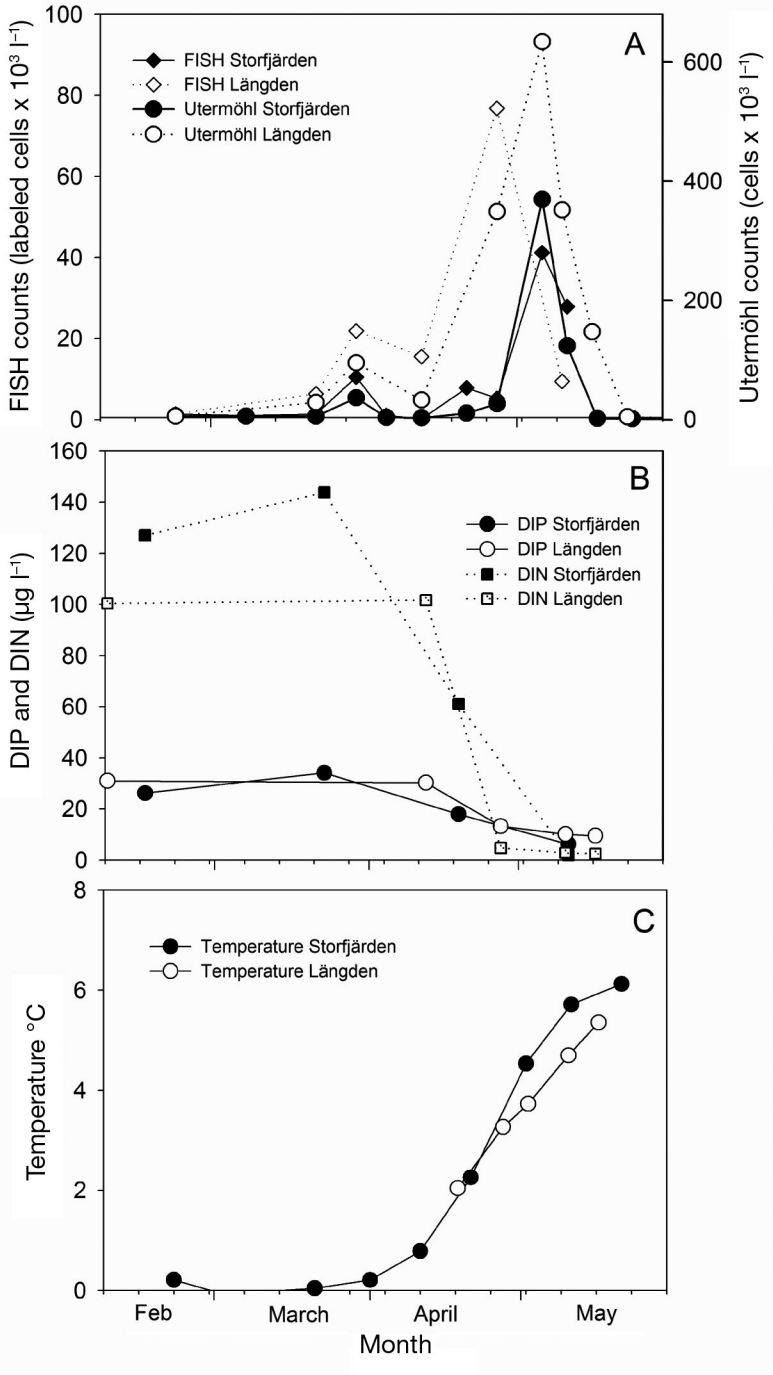

Fig. 3. (A) Succession of *Biecheleria baltica* (fluorescence *in situ* hybridization (FISH) counts) and the *Scrippsiella/Biecheleria/Gymnodinium* complex (Utermöhl counts) at offshore station Längden and inshore station Storfjärden in the Gulf of Finland during spring 2005. FISH data from Storfjärden on 6 May and Längden on 10 May were identified as outliers in Fig. 4. (B) Dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) concentrations at Storfjärden and Längden during spring 2005. (C) Temperature at Storfjärden and Längden during spring 2005

DISCUSSION

Probe selection and specificity

In this study, a FISH assay was developed for *Biecheleria baltica* to allow species detection in field samples. Five of 6 probes targeting the LSU rRNA of *B. baltica* produced labeling of the target species, with highest signal intensities obtained for BbRNA3 and BbRNA4. Of these 2 probes, BbRNA4 showed the least cross-reactivity with the other tested species and readily labeled the cultured *B. baltica* cells at optimized hybridization conditions. In the initial trials, BbRNA4 exhibited cross-reactivity with *Heterocapsa triquetra* and *Scrippsiella hangoei*. However, this cross-reactivity was eliminated by applying higher formamide concentration and temperature during hybridization, as suggested by Pernthaler et al. (2001).

The BbRNA4 probe cannot be considered species specific, as the sequence, when tested with BLAST, showed a 100% match with several closely related species, such as *Biecheleria pseudopalustris*, *Polarella glacialis*, *Protodinium simplex*, *Gymnodinium corii*, and *G. beii*. However, given the lack of general sequence variability of the available gene region (D1/D2) for these species, and the fact that except *P. simplex*, none of the species in question is expected to be present in the Baltic dinoflagellate community (Hällfors 2004), the probe is applicable for the Baltic Sea plankton. Indeed, *B. pseudopalustris* is a freshwater species, *P. glacialis* is confined to the polar oceans (Montresor et al. 1999, 2003), *G. corii* to the

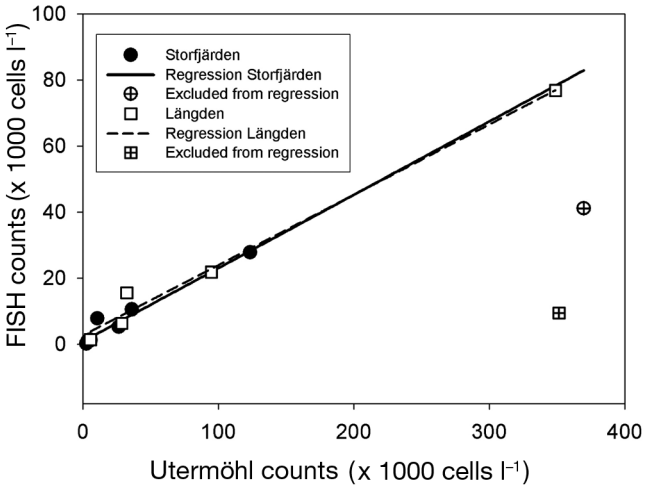

Fig. 4. Regression of fluorescence *in situ* hybridization (FISH) and Utermöhl cell counts at station Längden ($y = 0.2128x + 2.6436$, $R^2 = 0.9867$, $p < 0.001$, $n = 5$) and Storfjärden ($y = 0.2219x + 0.8280$, $R^2 = 0.9517$, $p < 0.001$, $n = 9$). Two samples from late May are omitted from the regression due to cell count complications

Adriatic Sea (Schiller 1928), and *G. beii* is symbiotic (Spero 1987). Nevertheless, the possibility of introduction of these species to the Baltic Sea in the future cannot be excluded given ongoing climate changes and the discharge of ballast water, and must be considered when applying the probe. *P. simplex*, on the other hand, is unlikely to be confused with *B. baltica* in a whole cell hybridization assay since it is very small in size (6 to 9 µm), even when compared to the small cells of *B. baltica* that are differentiated in connection with cyst formation (Kremp et al. 2009).

Probe signal and life-cycle transitions

An important issue to consider in probe-based detection for monitoring of phytoplankton is potential effects of physiological changes on the labeling quality. Nutrient status, growth phase, and life-cycle transformations may modify probe binding and consequently signal strength (Anderson et al. 1999, Sako et al. 2004, Touzet & Raine 2007). During the bloom, *Biecheleria baltica* is expected to undergo physiological changes, the most significant being the transformation of a large part of the population to resting cysts (Kremp & Heiskanen 1999). We therefore experimentally evaluated whether labeling characteristics would be affected by encystment. Cysts and life-cycle stages involved in the encystment process were observed in both the control and low P treatment. However, the labeling efficiency in the low P treatment was significantly lower than in the control treatment, suggesting that the observed effect might have been due to nutrient limitation, affecting cell biochemistry, rather than life-cycle transformations. These results are in agreement with the study of Anderson et al. (1999). When comparing cells from early exponential phase (Day 7) and encystment phase (Day 29), labeling efficiency in both the control and low P treatment was found to be significantly lower on Day 29. These results concur with the observations of Touzet & Raine (2007), where cells of *Alexandrium andersoni* and *A. minutum* from the late stationary phase, which usually involves life-cycle transformations, had a weaker fluorescence signal. Despite a significant decrease in labeling, its efficiency was still relatively high (>90%) at the end of the experiment; this is possibly a reflection of the low fraction (9 to 10%) of large cells, i.e. potential planozygotes, in the cultures on Day 29. Labeling efficiency may be expected to decrease considerably when the fraction of planozygotes is high.

Reduced labeling efficiency in encysting or aging cell populations may have implications for the monitoring of *Biecheleria baltica* population dynamics using FISH. During the period when vegetative cells are transformed to resting cysts, and planozygotes constitute a large fraction of the population as observed earlier for this species (Kremp & Heiskanen 1999), abundances of *B. baltica* may be underestimated. During the field study, a change in probe binding quality was observed. While the signal of the BbRNA4 probe was strong and distinct in the samples from March and April, the labeling became less clear from early May. It is possible that the loss of signal is due to physiological differences at a late bloom stage, when cells can be expected to have decreasing rRNA content due to nutrient limitation or other growth-limiting factors. The decline of the *B. baltica* population in mid- to late May corresponded to the near depletion of nitrogen, which possibly caused nutrient limitation in *B. baltica* cells and may have led to the weakening of the probe signal. In a study by Anderson et al. (1999), the probe signal was weaker when cells were subjected to nitrogen limitation compared to phosphorus limitation. Depending on the level of nutrient deficiency, the probe signal might be too weak for visual detection or the probe may fail to hybridize at all. Moreover, a weaker fluorescence signal may also be caused by variations in cell permeability or accessibility for the probe due to changing physiological conditions (Anderson et al. 1999, 2005). As the time when the labeling quality deteriorated coincided with the appearance of resting cysts, indicating ongoing life-cycle transitions, the weak fluorescence signal may also be a result of zygote formation and encystment. The significant reduction of labeling efficiency in the life-cycle experiment supports this interpretation.

Underestimation of cell densities with FISH

FISH counts of cultured material without the concentration steps necessary for field samples were 80% of conventional cell counts. In a study by Touzet & Raine (2007), this part was as high as 95%. Since the actual hybridization procedure involves repeated washing and filtration steps, some loss of cells is expected compared to the traditional quantification method where cells are counted directly from a settled sample. To some extent, this loss might also have been caused by cells that are recovered on the filter but fail to exhibit strong enough fluorescence compared to the background noise; this may be due to life-cycle changes, leading to lower RNA levels and/or impermeability of the cell wall, or disruption of cells leading to loss of RNA (Scholin et al. 1999).

When applying the FISH assay to sea water samples (not naturally containing the species complex) spiked with *Biecheleria baltica* at low concentrations, to simulate the natural concentrations of the *Biecheleria*/*Scrippsiella*/*Gymnodinium* complex in spring, we found that FISH counts were only 30% of the Utermöhl counts. John et al. (2003) also reported a substantial cell loss after hybridization of field samples. The major reasons for the low detection efficiency are most likely the screening and concentration processes for FISH involving several sieving and washing steps, resulting in considerable cell loss compared to the processing of samples for the Utermöhl method. Chemical matrix effects, caused by e.g. humic substances, might also have influenced the outcome by inhibiting the molecular procedure. In addition to this, cells in natural samples may sometimes be clumped or obscured by detrital particles, making the detection of target cells difficult (Scholin et al. 1999, John et al. 2003).

The large deviation from the Utermöhl counts found in the spiking experiment was reflected in the field data. Higher cell numbers obtained by the Utermöhl method were expected here because these counts include *Scrippsiella hangoei* and *Gymnodinium corollarium* besides *Biecheleria baltica*. According to the regression between Utermöhl and FISH counts, the *B. baltica* population accounted for approximately 20% of the *Scrippsiella*/*Biecheleria*/*Gymnodinium* complex throughout the entire sampling period, at both sampling stations. However, as the spiking experiment revealed a mean cell recovery of 30%, the low field abundances reflect the losses due to complex sample processing involved in the FISH assay, rather than a low contribution of *B. baltica* to the species complex. Nevertheless, during the end of the spring bloom when nitrogen limitation prevails, the number of detected cells might be even lower due to the low amount of rRNA available for hybridization. If compensated for the cell loss, *B. baltica* accounts for a large fraction of the species complex, indicating that it is the dominating species.

Biecheleria baltica bloom dynamics as revealed by FISH

The FISH data showed a characteristic pattern of bloom development at both sampling stations. *Biecheleria baltica* formed 2 peaks during the bloom period: the first in late March, shortly before ice break-up, and the second in late April/early May. This agrees with earlier observations for the *Scrippsiella*/*Biecheleria*/*Gymnodinium* complex (previously referred to as *Scrippsiella hangoei, Woloszynskia halophila*, or the *Scrippsiella*/*Woloszynskia* complex), which reported a first biomass peak under the ice in late winter and a second one later in spring (Kremp & Anderson 2000, Spilling 2007). The presence of an ice cover in early spring appears to favor the motile dinoflagellates by yielding stratified conditions (Margalef 1978). It has been suggested that under-ice blooms provide the dinoflagellates a head-start in competition with co-occurring diatoms and supports later bloom formation (Kremp et al. 2008). The decline of the first peak is not related to nutrient limitation, since inorganic nutrient concentrations are still high at that time. Furthermore, there was no evidence of cyst formation in late March, another factor that can lead to a decrease in cell abundances. At the time of the first decline, water temperatures were between 0 and 1°C and only beginning to rise. A significant increase in temperature that would induce mass encystment (Kremp et al. 2009) occurred only later in April. It is likely that the under-ice population dispersed due to vertical mixing and horizontal advection upon ice break-up. The dynamics of the second bloom are more likely regulated by nutrient availability and life-cycle processes. Inorganic nutrient levels were nearly depleted at the time of the second abundance peak, and the presence of numerous cysts during the late bloom stage indicates that large parts of the population were encysting as reported earlier for *B. baltica* bloom populations (Kremp & Heiskanen 1999). The spring bloom field study revealed higher cell concentrations of *B. baltica* at Längden (~22 000 cells l^{-1}), the offshore sampling station, compared to the inshore station Storfjärden (~10 000 cells l^{-1}). Jaanus et al. (2006) found that the *Scrippsiella*/*Woloszynskia* complex occurred at lower concentrations in coastal areas that are influenced by freshwater inflows. As the salinity differences between the stations in our study were minor (less than 0.5), the difference in abundance might be due to other factors. Our FISH data confirm the general bloom pattern

unambiguously for *B. baltica* and suggest that this species is a major player that determines the so far unresolved dynamics of the *Scrippsiella/Biecheleria/Gymnodinium* complex.

CONCLUSIONS

We developed a FISH assay that allows unambiguous distinction of *Biecheleria baltica* in the spring dinoflagellate community. When applying the method in a field survey, we found that the bloom dynamics of *B. baltica* follow the same trend as earlier established for the *Scrippsiella/Biecheleria/Gymnodinium* complex. However, the assay considerably underestimates cell numbers of *B. baltica* in the field and needs to be complemented with traditional cell counting methods. In combination with the latter, FISH helps to provide realistic estimates of the *B. baltica* population without potential bias due to the presence of morphologically similar *Scrippsiella hangoei* and/or *Gymnodinium corollarium*. When relating the FISH-derived cell concentrations to light microscopy data and taking the abundance underestimation due to cell losses into account, our field data suggest that *B. baltica* is the major component of the complex at the southwest coast of Finland. Physiological changes due to nutrient limitation and life-cycle transformations affect the labeling efficiency, which in addition to the sample processing losses may compromise the application of the FISH assay. Further investigation on the relative contribution of *B. baltica,* and other morphologically similar species, to the phytoplankton community is needed to gain a better understanding of the ecological role of spring bloom dinoflagellates in the Baltic Sea.

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Appendix 1. Effects of deleting different high values on the regression parameters of Fig. 4

Station	Data points (n)	Slope	Intercept	R^2	Adj. R^2	p
Längden	6	0.106	6.661	0.383	0.229	0.190
	5 (–10 May)	0.213	2.644	0.987	0.982	<0.001
	4 (–10 May, 27 April)	0.216	2.534	0.806	0.710	0.102
	5 (–27 April)	0.006	10.286	0.011	–	0.864
Storfjärden	10	0.113	3.059	0.890	0.876	<0.001
	9 (–6 May)	0.222	0.828	0.952	0.945	<0.001
	9 (–11 May)	0.107	2.210	0.959	0.954	<0.001
	8 (–6 and 11 May)	0.262	0.385	0.726	0.680	0.007
	7 (–30 March, 6 and 11 May)	0.223	0.642	0.432	0.319	0.108

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