Evidence has accumulated during the last decade showing that many established diatom morpho-species actually consist of several semicryptic or truly cryptic species. As these species are difficult or even impossible to differentiate by microscopic analysis, there is virtually no information on how they behave in natural environments. In this study, we developed a quantitative real-time PCR (qPCR) assay using TaqMan probes targeted to the internal transcribed spacer 1 (ITS1) to assess the spatial distribution and seasonal dynamics of an important component of the microphytobenthos of intertidal sediments. *Navicula phyllepta* Kützing is a brackish-marine morpho-species with a cosmopolitan distribution. Axenic clones of this species were isolated from natural assemblages of benthic diatoms at different intertidal stations in the Westerschelde estuary (The Netherlands). At least two distinct semicryptic species of *N. phyllepta* were present, as shown by differences in the quantity of DNA per cell, the ITS1 sequences and the copy number of ITS per cell. DNA and chl a concentrations extracted from sediment surface samples were closely correlated, showing that the DNA used for subsequent analysis mostly belonged to the microalgal community. The results of real-time qPCR from sites throughout the estuary and over several seasons agreed well with microscopic counts. Additionally, the seasonal pattern of the two forms of *N. phyllepta* showed an overlapping, but unique distribution along the estuary.

Key index words: diatoms; estuary; *Navicula phyllepta*; niche differentiation; real-time qPCR; semicryptic species

Benthic diatoms are one of the most important groups of photoautotrophic microorganisms in estuarine intertidal sediments where they play a major role in ecosystem functioning (Underwood and Kromkamp 1999). Several investigations have shown that benthic diatoms are an essential component of the food web of intertidal mudflats (Middelburg et al. 2000) as well as of the water column after re-suspension (Lucas et al. 2000). Many benthic diatom taxa are well adapted to fluctuating environmental conditions (Admiraal et al. 1984, Colijn and de Jonge 1984), which might explain their cosmopolitan distribution (Round 2004).

The growth rate and the rates of carbon fixation of benthic diatoms can equal those of pelagic species (Barranguet and Kromkamp 2000, Brandini et al. 2001). The presence of benthic biofilms may control the rate of nutrient exchange between the sediment and the water column (Sündback et al. 2000). Moreover, biofilms of benthic diatoms have been shown to contribute to the stabilization of estuarine sediment surfaces by causing an increase of the erosion threshold (Tolhurst et al. 2003).

Biofilms of microphytobenthos in intertidal mudflats of the Westerschelde estuary (the Netherlands) are generally dominated by raphid, motile pennate diatoms (epipelic diatoms) (Muylaert et al. 2002, Forster et al. 2006). These diatoms have been shown to belong to a monophyletic group of organisms (Medlin and Kaczmarska 2004, Sorhannus 2004) that appeared in the middle of the Eocene about 55 million years ago (Medlin et al. 1993). The majority of the epipelic diatoms in temperate intertidal mudflats belong to the order of Naviculales (Montani et al. 2003, Haubois et al. 2005). The microscopic identification of *Navicula* species is cumbersome because of the morphological plasticity inherent to their life cycle and because some characteristics can only be discerned by scanning electron microscopy (Cox 1997, 1998, Mann 1999). The morpho-species *Navicula phyllepta* Kützing is often a key species in intertidal mudflats. It can reach 60%–75% of the biomass of the total microphytobenthic community.
Ribosomal genes are frequently used for phylogenetic reconstructions. The nuclear ribosomal operon of diatoms has the structure 18S rDNA–ITS1–5.8S rDNA–ITS2–28S rDNA (Zechman et al. 1994) and may be present in multiple copies (Armbrust et al. 2004). While both 18S and 28S rRNA genes have been used for the reconstruction of the phylogeny of diatoms, the ITS region, comprising ITS1–5.8S rRNA gene–ITS2 has been proven to be useful for molecular analyses at the species level or beyond. Additionally, ITS has been used to resolve intra- and interspecific relationships of a variety of eukaryotes, including diatoms (Zechman et al. 1994, Behnke et al. 2004, Orsini et al. 2004), and the phylogenetic relationships between populations and their biogeographical repartition (Bakker et al. 1992, Kooistra et al. 1992). The broad ecological range of *N. phyllepta* suggests that this species complex may be composed of more than one taxon. Sequence analysis of the ITS from a variety of isolated strains demonstrated that the morpho-species *N. phyllepta* actually consists of two well-separated clusters which can be regarded as semicryptic taxa as they show subtle but stable morphological differences such as size, difference in valves width and stria density (B. Vanelslander et al. unpublished data).

In this study, a real-time quantitative PCR (real-time qPCR) assay using primers targeting part of the ITS1 region and Taqman® probes was developed in order to measure the abundance of the two semicryptic forms of *N. phyllepta* along the salinity gradient in the Westerschelde estuary. We determined their distinct spatial and seasonal distribution patterns along the estuary and compared the results with microscopic counts. Real-time qPCR has been applied in environmental studies to quantify the number of copies of a specific gene and to estimate the number of cells of a particular microorganism containing the targeted gene. It had been also used to quantify the number of transcripts of the target gene as a measure of the potential metabolic activity coded by that specific gene by using reverse transcriptase PCR in an initial step. These approaches have been successfully used in ecological studies on diatoms (Leblanc et al. 1999, Wawrik et al. 2002, Wei et al. 2004), dinoflagellates (Bowers et al. 2000, Galluzzi et al. 2004), cyanobacteria (Becker et al. 2000, Suzuki et al. 2000), and bacteria (Labrenz et al. 2004, Skovhus et al. 2004). Quantitative PCR offers all the advantages of conventional PCR, such as high sensitivity (Becker et al. 2002, Newby et al. 2003), reproducibility, and specificity.

**MATERIALS AND METHODS**

**Sampling sites and diatom isolation.** Microphytobenthic biomass and biodiversity were measured on several occasions at three intertidal locations, Appelzak (A), Biezelingsche Ham (B), and Paulina Polder (P), from oligohaline to polyhaline conditions in the Westerschelde estuary, The Netherlands (Fig. 1). At each location, high-shore (A1, B1, P1) and mid-shore (A2, B2, P2) stations were selected on the exposed mudflats. Shore heights of the stations relative to lowest and highest tidal levels were determined by reference to a digital elevation model of the estuary, and confirmed by direct observation of the timing of emersion and immersion periods.

![Map of study sites](image)

**Fig. 1.** Location of the study sites in the Westerschelde estuary (The Netherlands). Each site had two stations: high shore A_1_ (51°23′ 01N; 004°14′ 32E), B_1_ (51°26′ 40N; 003°55′ 28E) and P_1_ (51°20′ 59N; 003°43′ 51E), and middle shore A_2_ (51°25′ 00N; 004°14′ 19E), B_2_ (51°26′ 36N; 003°55′ 43E), and P_2_ (51°21′ 07N; 003°43′ 45E).
Characteristics of the sites are listed in Table 1. Each station was sampled nine times between April 2002 and September 2003.

In 2002, diatoms were isolated from all six stations. Biofilm samples were suspended in seawater from the same location and this suspension was subsequently spread on 1.5% agarose plates (Bacto Agar, Brunschwig Chemie, Germany) amended with F/2 growth medium (Guillard and Ryther 1962). After 2–4 weeks of incubation at 17°C at a 14:10 light:dark (L:D) cycle (light source Cool White 36W compact fluorescent tubes, Philips, The Netherlands) at 80–120 μmol photons m⁻² s⁻¹, individual colonies were picked and sub-cultivated on agarose plates as well as in liquid medium (f/2 growth medium). Dilution-plating and picking of colonies were repeated until axenic cultures were obtained (Table 2). This was checked by microscopic examination, and by the absence of bacterial growth on agarose plates amended with Bacto yeast extract and peptone.

**Microscopy.** The species composition in the microphytobenthos of the Westerschelde was determined from surface sediment samples (upper 2 mm, 18 cm²), removed with a contact corer (Ford and Honeywill 2002). At each station, within an area of approximately 25 m², five samples were taken and pooled. In order to visualize the ultrastructural features of the siliceous cell walls, aliquots of samples from each site were oxidized with a 1:1 mixture of hydrogen peroxide (30%) and acetic acid (100%). The oxidized material was mounted in Naphrax (PhycoTech, St. Joseph, MI, USA). Approximately 300 diatom valves (range, 277–336) were identified and counts were made by using a Leitz Diaplan microscope equipped with Differential Interference Contrast.

Cultivated diatoms were identified as described above. For quantitative extraction of DNA, cells of three cultures of two isolates of *Navicula phyllepta* (CCY 0222 and CCY 0212) were enumerated in a Bürker chamber. From each culture, four aliquots...
were counted four times and the average number of cells per milliliter were calculated.

**Chlorophyll a and DNA extraction.** From the same stations as described above, five individual surface sediment samples (upper 2 mm, 18 cm²) were removed with a contact corer. The samples were immediately frozen in liquid nitrogen, freeze-dried in the dark and stored at −80 °C until the analysis. Algal biomass was estimated from measurement of chl a. Pigments were extracted with 90% acetone from aliquots of 100 mg of freeze-dried sediment. Mechanical disruption was achieved using 1 mm beads in a Bead Beater for 20 s ensured the efficient release of pigments. Chlorophyll a was quantified by HPLC following the procedure described in Rijstenbil (2003) and expressed as the chl content per dry weight of sediment (μg chl a g⁻¹ dry weight).

Two samples were selected for each station for DNA extraction from the freeze-dried contact cores. Nucleic acids were extracted from approximately 100 to 200 mg dry sediment using the UltraCleen™ Soil DNA Kit (MoBio Laboratories, Solana Beach, CA, USA). An extra elution step was added to the manufacturer’s instruction to ensure quantitative removal of DNA from the purification columns (final volume 200 μL). The quality of the nucleic acid was evaluated on a 1% agarose gel (Sigma), run in 1 × TAE buffer and stained by SYBR Gold (Molecular Probes, Leiden, Zuidplecht, The Netherlands). DNA was quantified with the PicoGreen dsDNA quantification Kit (Molecular Probes) using a microplate reader (Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany).

A similar procedure was used to extract and quantify DNA content from *N. phyllepta* strain CCY 0222 and strain CCY 0212. Cells of three cultures of each strain were counted (see above), and two aliquots (2 and 4 mL of strain CCY 0222 and 1 and 2 mL of strain CCY 0212) of each culture were harvested by centrifugation (5000g for 10 min). DNA was extracted and quantified as described above.

**PCR Primers, TaqMan® probes, and real-time qPCR assays.** The entire ITS region was amplified using the forward primer in the end of the 18S rDNA (5'-GGAAGGT-GAAGTCGTAACAGG-3') and reverse primer in the beginning of the 28S rDNA (5'-CTTGTAGTTTCTTTCTTCC-3'). The PCR products were cloned in PCR II-TOPO vector TA cloning kit (Invitrogen, Breda, The Netherlands) using the Lightcycler FastStart DNA MasterHyb® Hybridization Probes and the Lightcycler FastStart DNA SYBR Green I (Roche Diagnostics). The kits were supplemented with our custom primers (final concentration, 0.5 μM each) and probes (final concentration, 0.2 μM each) and contained 5 μL of DNA in a final volume of 20 μL. The PCR program comprised an initial 10 min denaturation step at 95 °C and 40 cycles consisting of a 15 s denaturation step at 95 °C and a 1 min annealing/polymerization step either at 60 °C (cluster 1) or 63 °C (cluster 2) for both assay format, respectively. The two steps were connected by heating and cooling speeds of 10 °C and 20 °C/°s, respectively. Finally, the PCR products were cooled down to 40 °C. Each PCR run included a standard curve, established with a serial dilution of linear plasmids from cloned target sequences, ITS1, representative for cluster 1 (strain CCY 0222) or cluster 2 (strain CCY 0212), respectively. Controls without templates (H₂O) and with non-target DNA (10⁵ copies of the plasmid with the insert from the other cluster) were also included.

Quantification of the amount of target DNA in sediment samples was accomplished by measuring the threshold cycle (Cₜ, Heid et al. 1996) and using the standard curve to determine the starting copy number. The entire process of calculating Cₜ, preparing a standard curve, and determining starting copy number for unknown samples was performed by the software of the Lightcycler Software version 3.5 (Roche Molecular Biochemicals, Indianapolis, IN, USA).

**RESULTS**

**Efficiency of the DNA extraction from sediments and its relationship with chl a.** The efficiency of DNA extraction from mudflat sediments was tested with five freeze-dried contact cores collected at different sampling sites in the Westerschelde. The samples were chosen according to their silt content (minimum 5%, maximum 87%) and median grain size (21–220 μm). Three to four DNA extractions were processed from each sample using from 20 to 200 mg of dry sediment. In all cases, DNA recovery was proportional to the quantity of sediment treated. The correlation coefficients were slightly higher in sediments containing 50%–70% of sand ($r^2 = 0.99$, $P < 0.001$, $n = 6$) than with sequences of other *Navicula* species (Table 2). Primers and probes (Table 3) were designed using the program Primer Premier 5 (Premier Biosoft Int., Palo Alto, CA, USA) and the services of Tib-Molbiol (Berlin, Germany). TaqMan® probes were 5’-labeled with the fluorescent dye FAM™ and 3’-labeled with the quencher TAMRA™ (Tib-Molbiol).

Real-time qPCR assays were performed with the Lightcycler® (Roche Diagnostics, Almere, The Netherlands) using the Lightcycler FastStart DNA MasterHyb® Hybridization Probes and the Lightcycler FastStart DNA SYBR Green I (Roche Diagnostics). The kits were supplemented with our custom primers (final concentration, 0.5 μM each) and probes (final concentration, 0.2 μM each) and contained 5 μL of DNA in a final volume of 20 μL. The PCR program comprised an initial 10 min denaturation step at 95 °C and 40 cycles consisting of a 15 s denaturation step at 95 °C and a 1 min annealing/polymerization step either at 60 °C (cluster 1) or 63 °C (cluster 2) for both assay format, respectively. The two steps were connected by heating and cooling speeds of 10 °C and 20 °C/°s, respectively. Finally, the PCR products were cooled down to 40 °C. Each PCR run included a standard curve, established with a serial dilution of linear plasmids from cloned target sequences, ITS1, representative for cluster 1 (strain CCY 0222) or cluster 2 (strain CCY 0212), respectively. Controls without templates (H₂O) and with non-target DNA (10⁵ copies of the plasmid with the insert from the other cluster) were also included.

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**Table 3. List of primers and probes used for the real-time qPCR assays.**

<table>
<thead>
<tr>
<th>Clusters</th>
<th>Sequences</th>
<th>Tₘ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1 (CCY 0222)</td>
<td>DITS142F</td>
<td>TGGCCTCTATTTTGGG</td>
</tr>
<tr>
<td></td>
<td>DITS1127R*</td>
<td>AACTGTGGTTTTGGTTTT</td>
</tr>
<tr>
<td></td>
<td>ITS1R*</td>
<td>TGGCAAGTAGGCTTGAGCC</td>
</tr>
<tr>
<td>Cluster 2 (CCY 0212)</td>
<td>DITS15F</td>
<td>CACACCTTAYTCACAGATCAAT</td>
</tr>
<tr>
<td></td>
<td>DITS1113R*</td>
<td>CGTTTCAGATTGCCAGCA</td>
</tr>
<tr>
<td></td>
<td>ITS1F</td>
<td>CCGTGAAAYTGGCCTTACTC</td>
</tr>
</tbody>
</table>

Specific primers or probes are specified by an ‘*’.
in muddy sediments containing at least 70% of silt and clay ($r^2 = 0.94, P < 0.001, n = 14$).

As a measure for the biomass of the microphytobenthos, we determined the chl $a$ content of freeze-dried contact cores collected at the sampling sites during the nine sampling campaigns between May 2002 and September 2003. The DNA and chl $a$ content expressed on the basis of sediment dry weight varied between 2.8 and 80 ng g$^{-1}$ dry wt and 1.4–850 µg g$^{-1}$ dry wt, respectively. There was a positive correlation between the quantity of chl $a$ and DNA contents extracted from the same sediment sample ($r^2 = 0.78, P < 0.001, n = 95$; Fig. 2). The variations in DNA and chl $a$ contents depended on seasons and sediment characteristics. For both DNA and chl $a$ the highest values were observed in spring and in muddy sediments.

Specificity of the primers and efficiency of the real-time qPCR. Phylogenetic analysis of the 18S rRNA gene and the intergenic transcribed spacer region between 18S and 28S rRNA genes showed that $N$. phyllepta isolates from the Westerschelde formed two phylogenetic clusters (Fig. 3). The ITS1, which comprised 384 bp in cluster 1 and 441–448 bp in cluster 2, exhibited the highest sequence divergence (32%), including insertion/deletion mutations (indels; Fig. 4). The PCR-primers for specific detection of each cluster were designed so that each pair included one highly specific primer i.e. exhibiting at least 28% sequence divergence at the target position of the other cluster. Furthermore, they were selected for minimal tendency to form primer dimers. The PCR-primers selected for specific detection of genotypes assigned to clusters 1 and 2 produced 104 and 128 bp fragments of the ITS1, respectively. Specificity and efficiency of amplification was extensively tested using endpoint-PCR and real-time qPCR in two detection formats: SYBR-Green and TaqMan. The specificity of the qPCR was assayed using defined mixtures of

![Figure 2](image1.png)

**FIG. 2.** Relationship between DNA and chl $a$ extracted from sediment ($r^2 = 0.78, n = 95, P < 0.001$). Samples A1, A2, B1, B2 for April and May 2002–2003 and March 2003 (○), samples A1, A2, B1, B2 for October 2002–2003 and all the sampling dates for P1 and P2 (●).

![Figure 3](image2.png)

**FIG. 3.** Maximum likelihood (ML) tree for Internal Transcribed Spacer 1 (ITS1) showing the two clusters of $Navicula$ phyllepta. Bootstrap proportion values (≥50%) for ML (100 replicates) are shown above nodes. Branch lengths are proportional to the amount of substitutions.

$N$. phyllepta cluster 2

$N$. phyllepta cluster 1

$N$. gregoria I1P1-49

$N$. gregoria I1B1-37

$N$. gregoria B1-8

$N$. arenaria PS2-F1

$N$. arenaria CCY 0228

Cylindrotheca fusiformis AF289049
FIG. 4. Alignment of the ITS1 sequences of the two clusters of *Navicula phyllepta*. Strains CCY 0222 (DQ193556) and CCY 0212 (DQ193543) are in cluster 1 and cluster 2, respectively.

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Fig. 4. Alignment of the ITS1 sequences of the two clusters of *Navicula phyllepta*. Strains CCY 0222 (DQ193556) and CCY 0212 (DQ193543) are in cluster 1 and cluster 2, respectively.
reference plasmids, containing the cloned ITS1 fragments of *N. phyllepta* strain CCY 0222 (cluster 1) and strain CCY 0212 (cluster 2), respectively. The results showed no cross reactivity of the primers with non-target DNA. The two sets of primers were also applied to analyze the genomic DNA of *Navicula* strains isolated from the three locations in the Westerschelde as well as two reference strains from the Ems-Dollard estuary and the Colne River (see Table 2). An amplification product was only observed for strains that were identified as *N. phyllepta*. Moreover, in all strains tested only one set of PCR primers gave a positive result, and a fragment of the expected size, which allowed unequivocal assignment of all strains to either one of the clusters.

For reliable quantification, the amplification efficiency during PCR must be equal for the recombinant DNA used in the calibration curves and the DNA extracted from the investigated samples, and, for high sensitivity, the amplification efficiency should approach 100%. In the exponential phase of the PCR, the number of amplicons ($Y_n$) produced after $n$ PCR cycles is expressed as

$$Y_n = N_0(1 + e)^n$$

where $e$ is the amplification efficiency of one PCR step ($0 < e < 1$). In calibration curves performed with recombinant DNA reference plasmids and genomic DNA of *N. phyllepta* strains CCY 0222 and CCY 0212, $e$ was $1.08 \pm 0.08$ ($n = 6$, 0.13, 1), and $1.15 \pm 0.08$ ($n = 6$, 0.122, 1), respectively (in brackets, number of calibration curves, mean squared error, and regression coefficient). Because of this high efficiency, the fluorescent signal produced from 10 target sequences in a single TaqMan assay passed the threshold value within less than 35 cycles. Assays that exhibited lowered amplification efficiency were excluded from analyses.

**DNA content and number of ITS1 sequences per cell.** We determined the DNA content and the number of ITS1 copies per cell in two isolates of *N. phyllepta*, CCY 0212 and CCY 0222. Cells were harvested 15 days after inoculation at concentrations of $7.4 - 8.2 \times 10^6$ and $1.5 - 2.3 \times 10^6$ cells·mL$^{-1}$, respectively. The mean DNA content per cell was $0.15 \pm 0.02$ pg ($n = 11$) for strain CCY 0212 and $0.04 \pm 0.008$ pg ($n = 11$) for strain CCY 0222. The DNA samples (22) were used to estimate the number of ITS1 copies per cell in real-time qPCR assays. The number of copies per cell calculated from the regression equation (Fig. 5) was 92 copies per cell for CCY 0212 and 144 copies per cell for CCY 0222.

**Quantification of the two *N. phyllepta* clusters in the Westerschelde estuary.** The number of ITS1 sequences of each of the two ITS1 genotypes of *N. phyllepta* was determined by real-time qPCR in the DNA extracted from surface sediment samples, which were collected at three sites (six locations) along the salinity gradient of the Westerschelde estuary (Fig. 1). DNA extracted from a high-biomass sediment sample (samples with the highest chl $a$ content) was analyzed twice and the average values are shown in Figure 5. Owing to the patchiness of microphytobenthos in the field, DNA from a second sediment sample collected from the same station at the same date was analyzed. Even if the numbers of cells per square meter were different due to different biomass, a similar trend for both sediment samples was observed (data not shown).

The distribution of the two clusters differed significantly (Wilcoxon Matched pairs test, $T = 144$, $n = 54$, $P < 0.05$) along the estuary (Fig. 6). When both genotypes were present, cluster 1 showed a higher number of copies per square meter than cluster 2 (2-600 times more) except in April and May 2002 in P1 and April 2002 and 2003 in P2 (Fig. 6, P1, P2). Assuming that the number of ITS sequence copies determined for strains CCY 0222 (144 copies) and CCY 0212 (92 copies) are representative for all genotypes assigned to the same cluster, the cell number in surface sediment samples was calculated. Cell numbers of cluster 1 genotype varied from $8.5 \times 10^6$ at station P2 to $2.7 \times 10^{10}$ cells·m$^{-2}$ in station A2. No sample was below the detection limit. The cell density was significantly higher in Appelzak than in the Biezelinghe Ham and Paulina Polder sediments, and in April 2002 compared with the other sampling dates (MANOVA, $F_{2.107}, 8.107 = 11.09$ and $4.8, P < 0.01$). In contrast to cluster 1, the abundance of cluster 2 did not differ between sites (MANOVA, $F_{2.107} = 1.8$, $P = 0.155$). The cluster 2 genotype was not detected in February and September 2003 and reached maximum abundance of $3.9 \times 10^{9}$ cells·m$^{-2}$ in April 2002 at station P1 (Fig. 6B). There was no significant difference between high and middle shore sampling sites whatever the cluster (MANOVA, $F_{1.107} = 0.15$, $P = 0.15$ for cluster 1, $F_{1.107} = 2.78$, $P = 0.098$ for cluster 2).
Comparison of the number of cells calculated on basis of real-time qPCR and the relative abundance of N. phyllepta from microscope counts. Microscopic counting of the cleaned valves of epipelic diatoms showed a highest percentage of N. phyllepta in the microphytobenthos of Appelzak in May 2002: 24% and 37% at the sites A₁ and A₂, respectively. The percentages observed at the other locations (Biezelingsche Ham and Paulina Polder) varied between 0% and 10%, except in September 2002 when 19% of the valves counted at station P₁ were assigned to this species. As these values represent relative abundances within the total pool of diatoms, they were not directly comparable with cell densities derived from real-time qPCR. Using the average chl a per station and a cellular content of 5.3 pg of chl a per cell (De Jong and Admiraal 1984), we estimated the number of cells per square meter of N. phyllepta from the microscopic counts (Fig. 7). The values from the real-time qPCR and the microscopic counts were in the

![Comparison of the number of cells calculated on basis of real-time qPCR and the relative abundance of N. phyllepta from microscope counts.](image-url)
same order of magnitude, $5 \times 10^7$–$1.7 \times 10^{10}$ and $1.2 \times 10^7$–$6 \times 10^9$ cells $\cdot m^{-2}$, respectively. The population dynamic of *N. phyllepta* determined with the two methods showed a good agreement ($r^2 = 0.24$, $P<0.01$, $n = 48$), particularly in Appelzak (Fig. 7, A1, A2).

**DISCUSSION**

Recent investigations have identified epipelic diatoms as key species in the relationship between biodiversity and ecosystem function in intertidal environments (Forster et al. 2006). However, assessing the biodiversity of microalgae is not a trivial task. Determination at the species level is only possible by taxonomic experts but even then, different genotypes may reveal the same phenotype and vice versa. Owing to these difficulties molecular genetic approaches are more informative for following the diversity and distribution of microalgal populations. In this study, real-time qPCR was used to determine the diversity and distribution of *Navicula phyllepta*, a key species in intertidal mudflats.

The analysis of ITS sequences demonstrated that *N. phylepta* is a semicryptic species that is divided in at least two clusters that are difficult to identify by microscopy (B. Vanelslander et al. unpublished). The abundances of the two forms of *N. phyllepta* were quantified in samples obtained from intertidal mudflats along a salinity gradient in the Westerschelde estuary. The results showed that the two forms have a different distribution. Form 1 (i.e. ITS1 cluster 1) was dominant at the brackish sites and showed a recurrent seasonal pattern with highest densities in late spring. Form 2 (i.e. ITS1 cluster 2) was generally less abundant, occurred mainly at higher salinities, and only bloomed...
during spring of the first sampling year. A difference of distribution patterns of *N. phyllepta* caused by different organic waste and nutrient concentrations has been already described in estuaries (Peletier 1996, Thornton et al. 2002) but never related to different forms of the species. Cryptic or semicryptic species such as *N. phyllepta* have been reported for several marine organisms (Knowlton 1993, Darling et al. 2004), which may or may not be sympatric. The presence of the two forms in the same environment suggests that they occupy different niches and thrive under different environmental conditions such as temperature and nutrients, sensitivity to pollutants, chemical composition, or light stress conditions (Mann 1999). The simultaneous presence of semicryptic species in the same environment can also be explained by their life cycle strategies and the control exerted by predators or parasites (Orsini et al. 2004). In order to investigate the relationships, many clones of each cluster have to be isolated and tested for their properties and behavior under various conditions (Gallagher 1982, Rynearson and Armbrust 2004). Clones of *N. phyllepta* belonging to the two clusters have indeed been shown to differ in their growth responses to salinity (B. Vanelslander et al. unpublished data) in the laboratory. These results confirm the distribution pattern relative to salinity of the two forms in the estuary. However a wider survey of parameters is necessary to obtain a complete overview of the ecology of the two forms.

Trends in the distribution of *N. phyllepta* populations along the estuary analyzed by real-time qPCR have been compared with microscopic counting. In addition to being the only method available to enumerate organisms of cryptic species, this molecular technique is much faster compared to traditional taxonomic analyses and microscopic counting. It gives an accurate abundance of a certain genotype, which is not biased by an observer. In this study, the classic and molecular techniques gave rather similar results for the dynamic and the magnitude of *N. phyllepta* population. However, some discrepancies existed. The real-time qPCR showed a higher number of peaks for the population particularly at Biezelingsche Ham (B2) and Paulina Polder (P1, P2). These discrepancies could be explained not only by the heterogeneity of the samples, but also by using an average of chl a content per cell to convert the relative to absolute count for the microscopic data which does not take into account the variability between different species or different individuals. A good agreement between data from real-time qPCR and other methods has been observed for picoeukaryotes, Chlorophyta, and Mamiellales (Zhu et al. 2005) and *Alexandrium minutum* (Galluzzi et al. 2004). However, the comparison was not successful for *Aureococcus anophagefferens* (Popels et al. 2003). Although quantitative real-time PCR is attractive due to its sensitivity, speed, and the possibility of automation, a number of assumptions need to be considered. The various protocols for extracting DNA from microorganisms in soils or sediments are often not satisfactory. The reasons for this are that some microorganisms are difficult to break or that extracellular polymeric substances interfere. Co-extraction of humic acids is another problem that interferes with the quality of the extracted DNA. In recent years, commercial extraction kits for different sample materials have been significantly improved and are increasingly used because of their constant quality and hence the possibility for comparisons between different laboratories. The variability in efficiency, reproducibility, phylotype abundance, and composition of the microbial community strongly depends on the DNA extraction method applied (Martin-Laurent et al. 2001, Murny and Findlay 2004). The choice of the DNA extraction method is dependent on the type of soil, the characteristics of the sediment (Liphay et al. 2004) or the type of microorganism under study (Frostegard et al. 1999). In the present study, the extracted DNA was of high quality and could be directly amplified and quantified. A dilution step to decrease humic acid concentration was not necessary. Hence, the quantity of DNA obtained was not the limiting factor and did not affect the sensitivity of the method. The correlations in this study between the amount of recovered DNA and the quantity of extracted sediment, and between DNA and chl a contents in the sample indicated a high reproducibility and DNA extraction efficiency.

There is little known about DNA content per cell (DNA C-value) for diatoms and even less for marine benthic diatoms. It is also difficult to make any comparison with the values in the literature for other organisms (Vaulot et al. 1994, Veldhuis et al. 1997) as there is a 5000-fold range in DNA C-values in unicellular eukaryotic algae (see Table 1 in Cavalier-Smith 1978). The DNA content of *N. phyllepta* from form 2 was found to be in the same range as *Navicula pelliculosa* (Holm-Hansen 1969), whereas the DNA content per algal cell for form 1 was four times lower and appeared to be in the bottom end of the known values for algae (Cavalier-Smith 1978). However, the two forms of *N. phyllepta* followed the proportional relationship between cell size and DNA C-value observed for algae (Holm-Hansen 1969) and in eukaryotes in general (Gregory 2001). The division of the two forms of *N. phyllepta* was even more obvious in terms of numbers of copies of ITS1 in each cluster. The two forms differed from each other by 52 copies, but both were close to the regression line established in Zhu et al. (2005), which showed a positive relationship between rRNA gene copy number and cell length. The range of copy numbers is wide in unicellular eukaryotic algae from 1 for *Nannochloropsis salina* (Zhu et al. 2005) to 1000 in *Alexandrium minutum* (Galluzzi et al. 2004). The two forms of *N. phyllepta* presented copy numbers of 92 and 144, which are close to that of another Bacillariophyte *Nitzschia closterium* (Zhu et al. 2005). However, environmental heterogeneity during periods of asexual division, sexual recombination, and random mutation can generate diversity in microalgal species. The variability in ITS1
between the clusters and even within the cluster can be high for *N. phyllepta* and as a result the total number of cells of this species may be underestimated by a less efficient detection of target sequence that exhibit a sequence variation in the target sequences of primers or the probe. The real-time qPCR assay for *N. phyllepta* has been developed on the basis of 13 cloned ITS sequences from 11 strains isolated and cultivated from the Westerschelde, one strain from the Ems-Dollard estuary (NL) and one strain from the River Colne (UK). Although this number of strains is limited, they still probably represent the dominant clones for *N. phyllepta*, being isolated during a period of 2 years and during bloom events where the chance to obtain the more abundant genotypes is high. It is difficult to evaluate the clonal variability in ITS1 because of sexual crossing events.

In the last few years, the real-time qPCR appears to be a very powerful method to quantify organisms. It is a sensitive, specific and low time consuming technique for processing the analysis. However, the optimization of the assay can be laborious. For a species complex, one must take into account the number of clusters, their variability and the quantity of targeted gene for each cluster. This study showed two different clusters for *N. phyllepta*. They differed in DNA content and ITS gene copy number and sequences. The structure of the phylogenetic tree supports the conclusion that there is a potential interbreeding between the clones from the same cluster but not between the two clusters (Mann 1999, Behnke et al. 2004). Additionally, the real-time qPCR showed that their distribution differs along the estuary as well as their abundance. These new sets of data on *N. phyllepta* reinforces the idea (Thornton et al. 2002) that this key species for intertidal ecosystem should be revised, not only in term of genetic variations but also ecological preferences.

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