

## Spring bloom development, genetic variation, and population succession in the planktonic diatom *Ditylum brightwellii*

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### Abstract

We determined spatial and temporal variation in abundance, distribution, and genetic composition of the planktonic marine diatom *Ditylum brightwellii* in 21 water samples collected between February and June from five locations in Puget Sound and the Strait of Juan de Fuca, Washington. Four blooms with cell abundances of  $>1,000$  cells  $L^{-1}$  were detected during the sampling period. The blooms were comprised of two genetically distinct populations identified by analyzing up to three microsatellite loci from 707 individual cells. One population was detected at all locations from February to April and comprised three blooms. In May and June, it was gone, replaced by a second, genetically distinct population that comprised a different bloom. Both populations had high levels of diversity: on average, 94% of cells in each sample were genetically distinct. The two populations contained cells with identical 18S and 5.8S rDNA sequences and 1.1% sequence divergence at the internal transcribed spacer regions, which suggests that both populations were members of the same species. The populations detected in early and late spring were composed of cells with average valve diameters of 22 and 69  $\mu m$ , respectively. The population sampled in late spring, comprised of large-diameter cells, had a distinct genetic composition, indicating that it did not arise from a sexual reproduction event in the population sampled in early spring. Both populations were associated with significantly different exposures to solar irradiance and silicic acid concentrations, suggesting that environmental selection may regulate bloom dynamics of distinct populations.

Diatoms are unicellular photosynthetic algae that are able to undergo rapid increases in cell abundance under favorable conditions, forming large blooms that fuel ecosystems (e.g., Peinert et al. 1982) and drive biogeochemical fluxes (e.g., Brzezinski et al. 2001). In temperate regions, diatom blooms occur predominantly in spring and fall and are commonly composed of successive peaks in abundance of different species (e.g., Waite et al. 1992b). The succession of diatom species during bloom cycles is broadly explained by changes in nutrient concentrations,

grazing, and/or turbulence in models, such as that by Margalef (1978). Much of the spatial and temporal variation in species composition, however, is not explained by existing models. For example, in coastal environments, individual diatom species display different patterns of abundance from year to year (e.g., Smayda 1998) and from month to month (e.g., Waite et al. 1992b), often forming multiple blooms under different chemical and physical conditions (Waite et al. 1992a).

The ability of individual species of diatoms to bloom over a broad range of environmental conditions may be influenced by variation in the genetic composition and physiological capabilities of cells within a species. This variation can be partitioned in two ways: variation among individual clonal lineages and variation among populations of closely related clonal lineages. High levels of genetic and physiological variation have been observed among clonal lineages in both freshwater (e.g., Lewis et al. 1997) and marine (e.g., Gallagher 1982; Ryneerson and Armbrust 2000, 2004) diatoms. For example, several thousand genetically distinct clonal lineages were estimated to comprise a bloom of the diatom *Ditylum brightwellii* (Ryneerson and Armbrust 2005). The whole genome sequence of a *Thalassiosira pseudonana* isolate displays

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extensive sequence variation between homologous chromosomes, indicating that this species is also comprised of a large number of clonal lineages (Armbrust et al. 2004). Clonal lineages are assembled into populations, defined as groups of clonal lineages that interbreed and are genetically distinct from other populations (Hartl and Clark 1997). Genetically distinct populations within a single species can display different ranges of physiological capabilities (Gallagher 1982; Rynearson and Armbrust 2004) that may reflect adaptations to particular environments. The only study to date to examine the relation between genetic composition and the dynamics of multiple blooms found that different populations of *Skeletonema costatum* were detected in winter and summer (Gallagher 1980), suggesting that different populations may respond to and bloom under different environmental conditions. Because the genus *Skeletonema* contains many morphologically similar species (Medlin et al. 1991; Sarno et al. 2005), the multiple blooms of *S. costatum* may have been generated by multiple species.

Here, we determined spatial and temporal variation in abundance, distribution, and genetic composition of the centric diatom *D. brightwellii* to examine in more detail the role of genetic variation in the development of multiple blooms. Surface-water samples were collected from the inland estuaries of Puget Sound and the Strait of Juan de Fuca, Washington, which are deep (>200 m) and highly productive fjords (Ebbesmeyer and Barnes 1980). The two estuaries are separated by shallow sills (~60 m) that generate extensive mixing and recirculation of surface and deep waters. Sills also separate hydrographically distinct basins within the Sound where water samples were collected. The well-mixed Main Basin of Puget Sound is bounded by sills to the north and south. The relatively quiescent Hood Canal basin contains a single sill at the outlet. As a result, water parcels in the southern Main Basin can flush out of Puget Sound in about 1 week, whereas those in southern Hood Canal require several months to a year to flush out of the Sound (Ebbesmeyer and Barnes 1980). The diversity of mixing regimes allowed us to examine a broad range of environmental conditions over a relatively small geographic scale (100s of km). *D. brightwellii* was examined because it is known to form spring blooms at different times and in different basins (R. Horner pers. comm.; Waite et al. 1992b; Rynearson and Armbrust 2005) and because at least three genetically distinct *D. brightwellii* populations have been identified in the Puget Sound–Strait of Juan de Fuca region (Rynearson and Armbrust 2004). *D. brightwellii* is a diploid diatom that divides predominantly asexually and occasionally undergoes sexual reproduction (Steele 1965). DNA fingerprinting of *D. brightwellii* during the spring growth season allowed us to examine the influence of asexual and sexual reproduction on population dynamics and the influence of both environmental conditions and genetics on bloom formation.

## Methods

**Field sampling**—Monthly water samples (Feb–Jun 2000) were collected by seaplane from four sites in Puget Sound

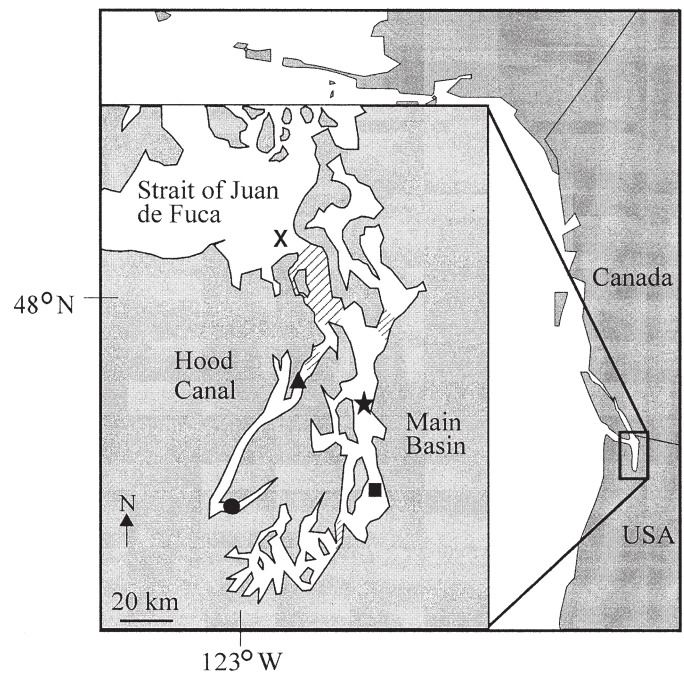


Fig. 1. Map of the northwest coast of North America. Inset indicates sampling site in the Strait of Juan de Fuca (X) and four sample locations in Puget Sound: northern Hood Canal (triangle), southern Hood Canal (circle), northern Main Basin (star), southern Main Basin (square). Relatively shallow (~60-m) sills (striped regions) define the boundaries of Hood Canal and the Main Basin.

and one site in the Strait of Juan de Fuca, Washington (Fig. 1). These samples were collected as part of the Washington State Department of Ecology marine water-quality monitoring program (Newton et al. 2002). Weather conditions prevented collection of samples from Hood Canal North in February and Main Basin North in April. Water was collected from the surface at all stations except Hood Canal South, where water was collected from 5-m depth. Up to 96 single cells of the marine centric diatom *D. brightwellii* were isolated from the 1-liter water samples and each isolate was cultured, harvested, and its deoxyribonucleic acids (DNA) extracted as previously described (Rynearson and Armbrust 2004). Differences in rates of survivorship of isolates from monthly water samples were tested using an ANOVA (Zar 1996).

Abundance and cell dimensions of *D. brightwellii* cells in each water sample were determined microscopically after concentrating the 1-liter water sample over a 20- $\mu$ m mesh net and preserving the sample with acid Lugol's iodine solution to a final concentration of 2%. Cells were counted and measured using a Zeiss Axiovert microscope. The entire fixed sample was settled in a counting chamber and the detection limit was one cell  $L^{-1}$ . No samples were fixed for Hood Canal South in February or from Main Basin North in March. Depending on *D. brightwellii* in situ concentrations, 20–500 cells were counted for abundance calculations and 3–69 cells were measured for cell-dimension analyses in each sample.

**Determination of genotypes**—Three microsatellite loci (Dbr4, 9, and 10) (Ryneckson and Armbrust 2000, 2004) were amplified from each single cell isolate in 10- $\mu$ L reactions containing 5–10 ng genomic DNA, 0.8 mmol L<sup>-1</sup> dNTPs, 3.13 mmol L<sup>-1</sup> MgCl<sub>2</sub>, 0.6 mg mL<sup>-1</sup> bovine serum albumin, 0.125 mmol L<sup>-1</sup> of each primer, 0.075 units *Taq* polymerase (Promega), and 1 $\times$  buffer (Promega). Each locus was amplified using polymerase chain reaction (PCR) profiles described in Ryneckson and Armbrust (2004). PCR products were analyzed on a MegaBACE 1000 automated sequencer (Amersham Biosciences) as described in Ryneckson and Armbrust (2004). Lengths of PCR products were determined using Genetic Profiler software (Amersham Biosciences) and used to construct a multilocus genotype for each isolate.

**Analysis of microsatellite data**—To determine the extent of clonal diversity ( $G : N$  ratio) in each water sample, the number of distinct three-locus genotypes ( $G$ ) was compared with the total number of three-locus genotypes analyzed ( $N$ ) (Ellstrand and Roose 1987). Genepop V3.3 (Raymond and Rousset 1995) was used to calculate gene diversity ( $H_e$ ) for each water sample at individual loci and over all loci using allele frequencies. Departures from Hardy–Weinberg equilibrium were determined using the Hardy–Weinberg exact test; heterozygote deficiency and excess were determined using the  $U$ -test (Raymond and Rousset 1995). Only water samples with 20 or more isolates were used in tests for departures from Hardy–Weinberg proportions and were calculated using Markov chain simulations of 5,000 iterations and 1,000 batches in Genepop V3.3 (Raymond and Rousset 1995). Differences in amplification success at each locus among samples and changes in single-locus  $H_e$  values over time were determined using a  $t$ -test for equality of means and a test for equality of variances (Zar 1996). The presence of null alleles and PCR amplification bias against large alleles were estimated using Micro-checker V2.2.0 (Van Oosterhout et al. 2003).

Analysis of population differentiation was restricted to the 12 water samples from which 20 or more isolates could be genotyped (Kalinowski 2002). Populations were defined by grouping all isolates within a water sample and calculating the estimator  $\theta$  (Weir and Cockerham 1984) of  $F_{ST}$  between different water samples. Genetic differentiation between samples from this study and with previously identified populations (Ryneckson and Armbrust 2004) was also determined. Genetix V4.02 (Belkhir 2001) was used to calculate pair-wise  $F_{ST}$  values, with significance levels determined using 1,000 permutation tests. A principle components analysis (PCA) was used to identify clusters of water samples based on  $F_{ST}$  values and was performed on the genotypic data using PCA-GEN V 1.2 (Goudet 1999). Significance of each axis in the PCA was determined using 1,000 randomizations of the genotype data. In all cases where significance was assigned to multiple tests,  $p$ -values for all pairwise comparisons were adjusted using the Dunn–Sidak multiple-comparisons method.

Cells from water samples with <20 isolates were assigned to a population using individual assignment tests calculated in GeneClass2 V2.0.b (Piry et al. 2004). The

Bayesian computation criterion of Rannala and Mountain (1997) and an alpha level of 0.01 were used to calculate the likelihood that a given isolate originated from a given genetically distinct population identified using  $F_{ST}$  methods.

**Ribosomal DNA and ITS sequencing**—Because nanogram quantities of DNA were extracted from individual isolates using the protocols described above, a whole genome amplification step using genomiphi (Amersham Biosciences) was added to ensure sufficient DNA was available for PCR amplification of 18S, 5.8S, and internal transcribed spacer (ITS) regions. For PCR amplification, whole genome amplified DNA (1 : 100 or 1 : 1,000 dilutions) was mixed with 0.1 mmol L<sup>-1</sup> dNTPs, 0.05 U  $\mu$ L<sup>-1</sup> Accuzyme DNA polymerase (Bioline), 1 $\times$  buffer (Bioline), and 3 pmol L<sup>-1</sup> each of forward and reverse primers. For 18S amplification, the universal 18SA and 18SB primers (Medlin et al. 1988) lacking the 5' restriction sites were used. The PCR consisted of a 2-min denaturation step at 95°C, 33 cycles of 94°C for 20 s, 70°C for 60 s, and 72°C for 2 min followed by a final 10-min extension at 72°C. For amplification of ITS I, the following primers were designed; 1645F, CTTATCATTTAGAGGAAGGT-GAAGTCGT; Dit5.8sR, CTGCGTTCCTCATCGTTG-TGG. ITS II was amplified with newly designed primers Dit5.8sF, CCACAACGATGAAGAACGCAGC and Dit28sR, AGTAACGGCGAGTGAAGCGG. The PCR consisted of a 2-min denaturation step at 95°C, 36 cycles of 94°C for 30 s, 67°C (ITS I) or 68°C (ITS II) for 30 s, and 72°C for 1 min followed by a final 10-min extension at 72°C. For each isolate, the amplification product from seven reactions (18S) or three reactions (ITS I and II) was pooled, electrophoresed through a 1% agarose gel, and the appropriately sized band was cut out of the gel. The DNA was extracted from the gel using a gel-extraction kit (Qiagen), concentrated, and used as template for cycle sequencing. For 18S rDNA genes, both strands were sequenced to completion using the primers 18SA and 18SB (Medlin et al. 1988); 18SC2, 18SE2, and 18SF3 (Ryneckson and Armbrust 2004); and 18SD (Armbrust and Galindo 2001). In addition, newly designed primers were TACCCAATCCTGACACAG (primer 18SC4) and AATTCCAGCTCCAATAGCGTA (primer 18SC5). Both strands of the ITS I and II and 5.8S regions were sequenced to completion. Sequencing was performed as described in Ryneckson and Armbrust (2004), and the resulting sequences were deposited in GenBank (accession numbers DQ329268–78).

The extent of the ITS I, 5.8S, and ITS II regions were determined by sequence alignment with full-length sequences of 18S rDNA from *D. brightwellii*, and the 5.8S and 28S rDNA from *Cylindrotheca* sp. (GenBank accession AF289049). Sequence divergence between ITS haplotypes was calculated using a two-parameter model of nucleotide substitution (Kimura 1980).

**Analysis of field data**—Differences in the size distribution of valve diameters between water samples defined as containing population A or B were determined using a two-

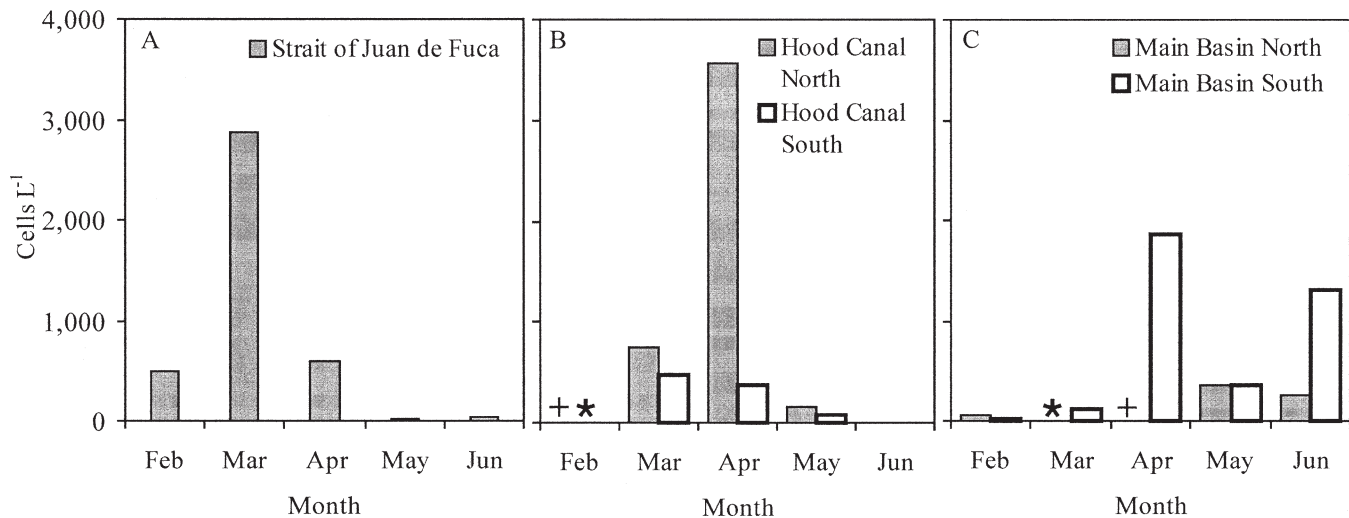


Fig. 2. Abundance of *D. brightwellii* cells between February and June, 2000 in (A) the Strait of Juan de Fuca, (B) Hood Canal North and South, Puget Sound, and (C) Main Basin North and South, Puget Sound. Asterisks indicate water samples without cell count data and crosses indicate months where no sample was collected because of inclement weather.

sample Kolmogorov–Smirnov test (Zar 1996). Cell biovolumes were estimated using mean valve diameter, mean cell length, and volume calculations for an equilateral prism.

Factor analysis was used to determine if either bloom events or genetically distinct populations were associated with any of the following environmental parameters: solar irradiance, temperature, salinity, or  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ , and  $\text{SiOH}_4$  concentrations. Temperature, salinity, and  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ , and  $\text{SiOH}_4$  concentrations were measured for each water sample by the Washington State Department of Ecology (Newton et al. 2002). Hourly solar irradiance exposure (global shortwave in  $\text{W m}^2$ ) for Seattle, Washington ( $47^\circ 40.80' \text{N}$ ,  $122^\circ 15.00' \text{W}$ ) between February and June 2000 was obtained from the National Oceanic and Atmospheric Administration integrated surface irradiance study ([www.atdd.noaa.gov/isis.htm](http://www.atdd.noaa.gov/isis.htm)). Solar exposure for cells in each water sample was approximated by calculating the average daily shortwave irradiance exposure over the 2-week time period prior to sampling. Principle components with eigenvalues larger than one were extracted using the varimax rotation method with Kaiser normalization and calculated in SPSS (SPSS Inc.). For each water sample, environmental data were regressed on the first two factors using the rotated component matrix. Two-sample Kolmogorov–Smirnov tests were conducted to determine if the regression scores for the first two factors were significantly correlated with water samples collected during bloom events (defined as abundance  $>1,000$  cells  $\text{L}^{-1}$ ) and nonbloom events (defined as abundance  $<1,000$  cells  $\text{L}^{-1}$ ) or with water samples representing genetically distinct populations.

## Results

**Abundance and distribution of *D. brightwellii***—The abundance of *D. brightwellii* cells between February and June 2000 ranged from below detection ( $<1$  cell  $\text{L}^{-1}$ ) to up

to 3,570 cells  $\text{L}^{-1}$  (Fig. 2A–C). Peak abundances occurred in March in the Strait of Juan de Fuca (Fig. 2A), in April in Hood Canal (Fig. 2B), and in April and June in the Main Basin (Fig. 2C). Both intrabasin and interbasin variation in cell abundance were detected within Puget Sound. *D. brightwellii* was always more abundant in the northern than the southern stations within Hood Canal, and Hood Canal displayed a higher peak abundance than the Main Basin. By June, cell abundance had dropped to below detection in Hood Canal but not in the Main Basin.

**Genetic diversity and population structure of *D. brightwellii***—A total of 1,195 single cells were isolated between February and June 2000. Survival of single-cell isolates from individual water samples averaged 67%, with survivorship ranging from 0% to 100% (Table 1). There was no significant relationship among rates of survivorship with month of sampling ( $p = 0.994$ ). Water samples with survivorship  $<50\%$  contained *D. brightwellii* in poor physiological condition (plasmolysis had occurred or cells contained few chloroplasts). Overall, 741 isolates survived, from which 707 could be genotyped (Table 1).

The three microsatellite loci used here were all polymorphic: 60 alleles were detected for Dbr9, 30 alleles for Dbr4, and 16 alleles for Dbr10. Locus Dbr4 could not be amplified from 325 isolates and null allele analysis indicated a general excess of homozygotes for most allele size classes, suggesting that this locus contained one or more null alleles that were not amplified because of mutation(s) in the microsatellite flanking sequence targeted by the primers. The number of isolates that could be amplified at locus Dbr4 differed significantly ( $p < 0.001$ ) between the 13 water samples collected before May (83% amplification success) and the 6 water samples collected in May and June (47% amplification success). No comparable relationship between time of cell isolation and amplification success was observed for loci Dbr9 (92% success rate) or Dbr10 (93% success rate).

Table 1. Overview of single-cell isolations, rDNA sequencing, and PCR amplification at three microsatellite loci. Clonal diversity is indicated by  $G : N$ , the proportion of novel three-locus genotypes. NA indicates that the calculation is not applicable.

Sample location	Date	No. cells isolated (survived)	No. ITS1, ITSII, and 5.8S rDNA sequences		No. 18S rDNA sequences	No. isolates Genotyped*	No. isolates			No. three-locus genotypes	$G : N$
			Dbr4†	Dbr9†			Dbr10†				
Main Basin South	09 Feb	14(12)	0	0	0	11	8	11	10	7	1
Main Basin North	09 Feb	16(15)	0	0	0	15	14	15	15	14	1
Strait of Juan de Fuca	09 Feb	96(2)	0	0	0	2	1	2	2	1	1
Hood Canal South	16 Feb	48(43)	0	0	0	43	40	43	43	40	0.9
Main Basin South	21 Mar	8(7)	1	1	1	7	6	6	6	5	1
Main Basin North	21 Mar	48(2)	1	0	0	2	2	2	2	2	1
Strait of Juan de Fuca	21 Mar	96(23)	2	1	1	22	19	22	21	18	0.94
Hood Canal South	27 Mar	80(64)	1	0	0	62	52	62	62	52	0.81
Hood Canal North	27 Mar	96(50)	0	0	0	48	41	48	48	41	0.95
Main Basin South	11 Apr	96(80)	0	0	0	79	66	78	78	65	0.97
Strait of Juan de Fuca	11 Apr	91(64)	0	0	0	63	52	62	63	51	0.82
Hood Canal South	18 Apr	65(40)	0	0	0	35	31	35	34	31	0.94
Hood Canal North	18 Apr	96(29)	1	0	0	29	19	28	28	19	1
Main Basin South	02 May	96(84)	1	1	1	79	14	61	76	12	0.83
Main Basin North	02 May	77(76)	1	0	0	74	5	58	71	5	1
Strait of Juan de Fuca	02 May	4(4)	0	0	0	3	2	3	3	2	1
Hood Canal South	16 May	0(0)	0	0	0	0	0	0	0	0	NA
Hood Canal North	16 May	20(0)	0	0	0	0	0	0	0	0	NA
Main Basin South	13 Jun	96(95)	1	1	1	90	9	73	76	6	0.83
Main Basin North	13 Jun	44(43)	1	0	0	36	1	34	10	0	1
Strait of Juan de Fuca	13 Jun	8(8)	1	0	0	7	0	5	7	3	1

\* Represents the number of isolates from which at least one locus could be PCR amplified.

† Indicates the number of isolates that could be amplified from each water sample.

No significant departures from Hardy–Weinberg proportions were detected in one water sample analyzed at locus Dbr9 (Strait of Juan de Fuca, April) and seven water samples analyzed at locus Dbr10 (Table 2). Significant heterozygote deficiencies ( $p < 0.05$ ) were observed for all other sample and locus combinations (Table 2). Monte Carlo analysis indicated that null alleles were a possible source of heterozygote deficiencies at locus Dbr9 in all samples except Main Basin South, June, and at locus Dbr10 in samples collected in May and June. Regression

analysis of allele-specific  $F_{IS}$  statistics on allele size (Wattier et al. 1998) did not indicate that PCR amplification bias against large alleles contributed to the observed heterozygote deficiencies ( $p > 0.05$ ).

The 374 isolates that could be genotyped at all three loci were used to compare clonal diversity of the different water samples. On average,  $94\% \pm 7\%$  of the cells in each water sample were composed of different genotypes (Table 1). The February Strait of Juan de Fuca sample was not included in determining the average proportion of novel

Table 2. Summary of observed heterozygosity ( $H_o$ ) and gene diversity ( $H_e$ ) at each locus for water samples with  $>20$  isolates. Asterisks (\*) indicate samples with significant heterozygote deficiency ( $p < 0.05$ ). NA indicates that no isolates could be genotyped.

Sample location	Date	Dbr4		Dbr9		Dbr10	
		$H_o$	$H_e$	$H_o$	$H_e$	$H_o$	$H_e$
Hood Canal South	16 Feb	0.38*	0.80	0.81*	0.91	0.33*	0.42
Strait of Juan de Fuca	21 Mar	0.32*	0.75	0.59*	0.86	0.43	0.45
Hood Canal South	27 Mar	0.37*	0.84	0.74*	0.87	0.31	0.43
Hood Canal North	27 Mar	0.41*	0.80	0.77*	0.89	0.46	0.41
Strait of Juan de Fuca	11 Apr	0.29*	0.79	0.84	0.89	0.49	0.51
Main Basin South	11 Apr	0.38*	0.80	0.71*	0.90	0.55	0.56
Hood Canal South	18 Apr	0.42*	0.76	0.54*	0.88	0.44	0.44
Hood Canal North	18 Apr	0.26*	0.76	0.71*	0.86	0.46	0.53
Main Basin South	02 May	0.21*	0.72	0.39*	0.92	0.24*	0.83
Main Basin North	02 May	0.20*	0.82	0.17*	0.92	0.30*	0.79
Main Basin South	13 Jun	0.67*	0.90	0.36*	0.92	0.32*	0.74
Main Basin North	13 Jun	NA	NA	0.18*	0.91	0.17*	0.60

Table 3. Extent of genetic differentiation between water samples ( $F_{ST}$ ). Bold numbers indicate significant  $F_{ST}$  values ( $p < 0.05$ ).

Sample location	Date	Hood Canal South		Strait of Juan de Fuca		Hood Canal North		Hood Canal South		Hood Canal North		Main Basin South		Main Basin North		Main Basin South		Main Basin North	
		16 Feb	21 Mar	27 Mar	27 Mar	11 Apr	11 Apr	18 Apr	18 Apr	18 Apr	18 Apr	02 May	02 May	02 May	02 May	13 Jun	13 Jun	13 Jun	13 Jun
Hood Canal South	16 Feb			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Strait of Juan de Fuca	21 Mar		0																
Hood Canal South	27 Mar		0	0	0	0.002	0	0	0	0	0	0	0	0	0	0	0	0	0
Hood Canal North	27 Mar			0	0	0.001	0	0	0	0	0	0	0	0	0	0	0	0	0
Strait of Juan de Fuca	11 Apr																		
Main Basin South	11 Apr																		
Hood Canal South	18 Apr																		
Hood Canal North	18 Apr																		
Main Basin South	2 May																		
Main Basin North	2 May																		
Main Basin South	13 Jun																		

genotypes because only one three-locus genotype could be obtained. A total of 306 genotypes were identified over the course of the study. Thirty-nine genotypes were detected more than once. One genotype was isolated a total of eight times: once in February and twice each in March and April from Hood Canal and once in March and twice in April from the Strait of Juan de Fuca.

$F_{ST}$  was used to examine genetic differentiation between the 12 water samples for which more than 20 individuals could be isolated (Table 3).  $F_{ST}$  can range from 0 (no genetic differentiation) to a theoretical maximum of 1 (complete genetic differentiation).  $F_{ST}$  values were calculated using only loci Dbr9 and Dbr10 because the amplification success of Dbr4 appeared to be dependent on when cells were isolated and thus was not a consistently informative marker. For 32 of 66 pairwise comparisons,  $F_{ST}$  values were significantly different from zero ( $p < 0.05$ ) and indicated the presence of two genetically differentiated populations that were independent of sampling location. One population consisted of cells collected in early spring, between February and April, and was designated as population A. The second population consisted of cells collected in late spring, between May and June, and was designated as population B. The pairwise  $F_{ST}$  between the two populations was 0.186 ( $p < 0.001$ ).

The two populations were characterized by unique alleles. For example, the most common Dbr10 allele composed 37% of the alleles from population B, but none from population A (data not shown). Overall, 63%, 62%, and 56% of the alleles at loci Dbr4, Dbr9, and Dbr10, respectively, were found in only population A or B, but not both.

*Assignment of individual diatom cells to genetically distinct populations*—Genetic-assignment tests conducted on cells from the seven water samples with <20 isolates genotyped revealed that cells collected between February and March, regardless of location, were more genetically similar to population A than B (Fig. 3). One of three cells collected in May and all cells collected in June from the Strait of Juan de Fuca were more genetically similar to population B. The single-cell assignment technique was useful when *D. brightwellii* abundance was low (<20 cells  $L^{-1}$ ), and few isolates could be obtained allowing an analysis of population structure during both bloom and nonbloom periods.

*Comparison with previously identified D. brightwellii populations*—The genetic signatures of populations A and B were compared with three previously identified populations in the Puget Sound region (Rynearson and Armbrust 2004). There was no significant genetic differentiation between population A and a population sampled repeatedly inside Puget Sound in November 1997, August 1998, and March 2000, indicating that population A can also be found during the fall. All other pairwise comparisons between populations A and B and the previously identified populations indicated significant genetic differentiation ( $p < 0.05$ ), with  $F_{ST}$  values ranging from 0.05 to 0.25. A total of four genetically distinct populations were

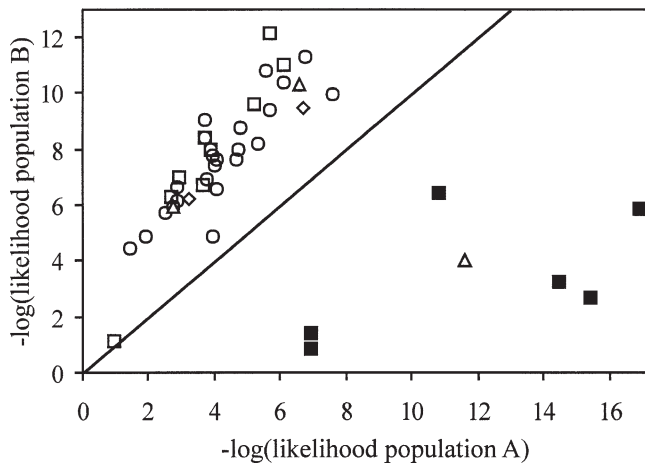


Fig. 3. Results of genetic assignment tests conducted on cells from seven water samples with <20 isolates each. Symbols represent cells isolated from the Main Basin (North and South) in February (open circles) and March (open squares) and the Strait of Juan de Fuca in February (open diamonds), May (open triangles), and June (filled squares). The line terminating at the origin indicates the location where the genetic likelihoods of belonging to either population A or B are equal. The area above the line indicates a greater likelihood of belonging to population A, and the area below the line indicates a greater likelihood of belonging to population B.

identified; one comprised population A and the population previously identified from Puget Sound waters, one comprised population B, one comprised a population collected from Strait of Juan de Fuca waters in August 1998, and one comprised a population collected from Strait of Juan de Fuca waters in September and October 1998. PCA of pairwise  $F_{ST}$  values was used to determine how isolates from the different water samples collected in this study were clustered with previously identified populations (Fig. 4). The first two axes of the PCA explained 85% and 5% of the variation in  $F_{ST}$ , respectively. Variation along the first axis of the PCA (PC1) was significant ( $p = 0.001$ ), but not along the remaining 10 axes ( $p > 0.05$ ).

**Ribosomal and ITS variation between populations**—The partial 18S rDNA sequences of randomly selected isolates from the early ( $n = 2$ ) and late ( $n = 2$ ) populations (Table 1) were identical. The ITS I and II regions of randomly selected isolates from the early ( $n = 6$ ) and late ( $n = 5$ ) populations (Table 1) were 638 and 276 base pairs (bp) in length, respectively. Ten bp differed among the 11 isolates sequenced (Table 4) and allowed two different haplotypes to be identified. Haplotype A was observed in all six isolates from population A and haplotype B was observed in all five isolates from population B. The Kimura two-parameter sequence divergence between haplotypes was 0.011. All 11 isolates had identical partial 5.8S DNA sequence.

**Morphological variation between populations**—Valve diameters of *D. brightwellii* cells ranged from 12 to 100  $\mu\text{m}$ . Samples defined as originating from populations A and B

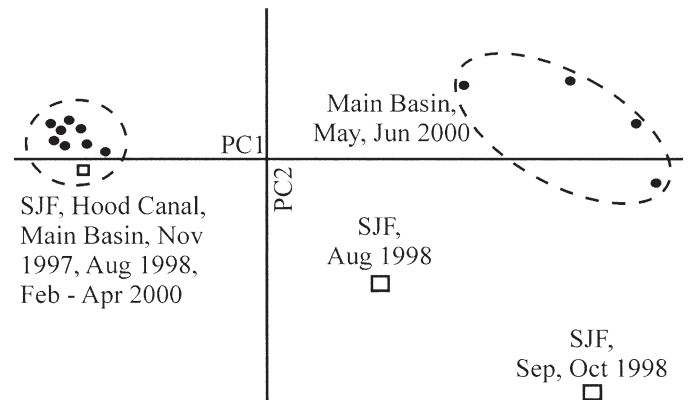


Fig. 4. PCA of genetic differentiation among the 12 water samples with >20 isolates and previously identified *D. brightwellii* populations. Because PC1 and PC2 explain 85% and 5% of the genetic variation among samples, respectively, the scale of axis PC2 has been exaggerated about 10 times relative to PC1. Filled circles represent water samples collected in this study and open squares represent populations identified in a previous study (Ryner and Armbrust 2004). The left dashed ellipse is defined as population A. Filled circles inside the left dashed ellipse represent water samples collected from the Strait of Juan de Fuca (SJT), Hood Canal, and the Main Basin between February and April 2000. The open square inside the left dashed ellipse represents previous samples of population A in Puget Sound sampled in 1997, 1998, and 2000. The right dashed ellipse is defined as population B. Filled circles inside the right dashed ellipse represent water samples collected from the Main Basin of Puget Sound in May and Jun, 2000.

contained cells with mean valve diameters <28  $\mu\text{m}$  and >60  $\mu\text{m}$ , respectively (Fig. 5). There was a significant difference in the distribution of valve diameters between populations A and B ( $p < 0.001$ ). Extreme values were recorded in six water samples and were defined as valve diameters that were more than three interquartile ranges larger or smaller than the interquartile range (the range containing at least 50% of the valve-diameter values). Extreme values in Hood Canal South in March, Main Basin North in May, and Main Basin South in June were characteristic of samples containing cells with valve diameters <28  $\mu\text{m}$ . In contrast, extreme values detected for the Strait of Juan de Fuca in March, Hood Canal North in April, and Main Basin North in June were characteristic of samples with mean valve diameters >60  $\mu\text{m}$ . Estimates of cell biovolumes were calculated using the mean-valve diameter for each population (22 and 69  $\mu\text{m}$ ) and the mean

Table 4. Sequence variation between isolates collected from populations A and B at the 18S, 5.8S, ITS I, and ITS II rDNA regions.

rDNA region	Length, bp (No. isolates sequenced)	No. bp differences among isolates	Nucleotide divergence*
18S	1,805(4)	0	0
ITS I	638(11)	7	0.011
5.8S	154(11)	0	0
ITS II	276(11)	3	0.011

\* Calculated from Kimura (1980).

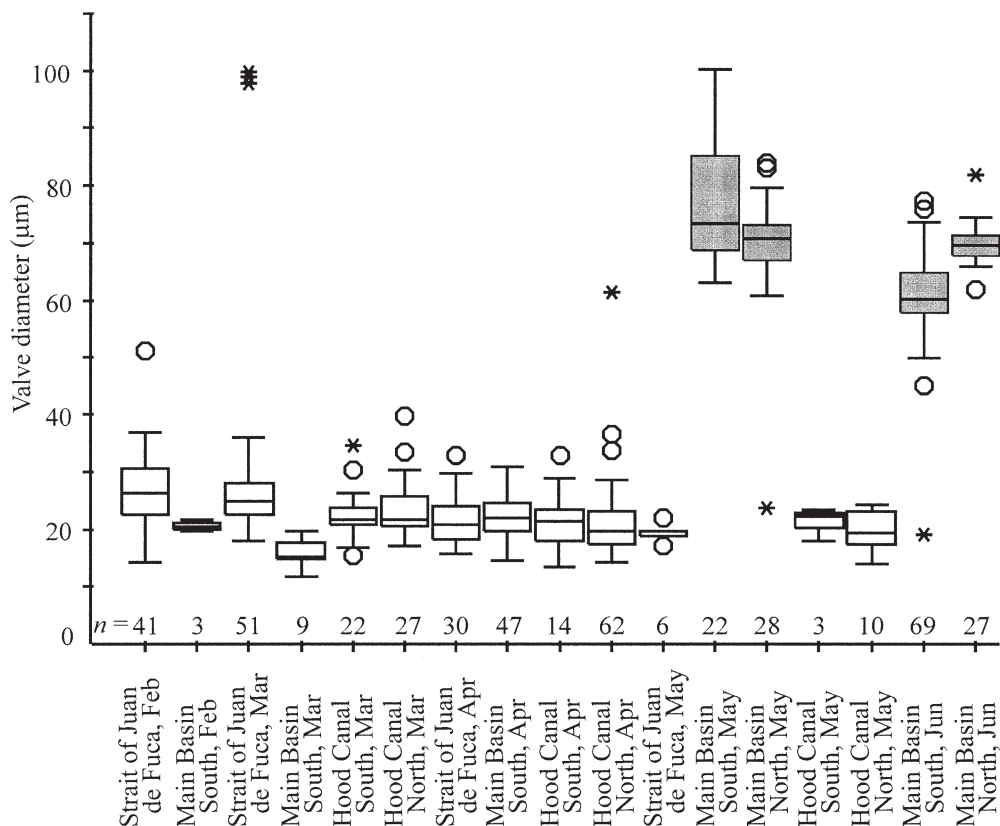


Fig. 5. Box plot of valve-diameter distributions of isolates collected between February and June. Number of cells ( $n$ ) measured in each water sample is listed above the X-axis. Boxes represent the interquartile range of valve diameters containing 50% of the values. Lines inside boxes represent the median and the whiskers extend from the highest to lowest values, excluding outliers and extreme values. Circles represent outliers (values between 1.5 and three box lengths outside the interquartile range). Asterisks represent extremes (values more than three box lengths outside the interquartile range). Grey shading inside boxes indicates samples that were composed of cells representing population B.

length for all cells ( $100 \mu\text{m}$ ). Populations A and B consisted of cells averaging  $20,960$  and  $206,100 \mu\text{m}^3$ , respectively.

*Environmental variation among blooms*—Between the first and last blooms observed, exposure to solar irradiance changed by a factor of two and concentrations of  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ , and  $\text{SiOH}_4$  changed by factors of 50, 5, and 4, respectively. Factor analysis was used to identify whether bloom events (abundance  $>1,000$  cells  $\text{L}^{-1}$ ) and nonbloom events (abundance  $<1,000$  cells  $\text{L}^{-1}$ ) or genetically distinct populations were associated with any of the following environmental parameters:  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ , or  $\text{SiOH}_4$  concentrations; solar irradiance; temperature; or salinity. The first four components of the factor analysis explained 41%, 38%, 9%, and 7% of the environmental variation, respectively. The remaining four components explained 5% of the environmental variation. Eigenvalues of the first two components were larger than one, indicating that these factors extracted at least as much as the equivalent of one original variable. Of the eight different environmental components analyzed, factor 1 was most

positively correlated with  $\text{PO}_4^{3-}$  and  $\text{NO}_3^-$  concentrations (0.956 and 0.936, respectively) and most negatively correlated with temperature ( $-0.443$ ). Factor 2 was most positively correlated with solar irradiance (0.961) and most negatively correlated with  $\text{SiOH}_4$  concentration ( $-0.859$ ). Water samples were spread broadly over the space defined by factors 1 and 2 (Fig. 6). There were no significant differences in regression scores of factor 1 between the bloom and nonbloom samples ( $p = 0.643$ ) or the two genetically distinct populations ( $p = 0.425$ ). Both populations appeared to tolerate a wide range of temperatures ( $8$ – $14^\circ\text{C}$ ) and concentrations of  $\text{NO}_3^-$  ( $0.1$ – $28.7 \mu\text{mol L}^{-1}$ ) and  $\text{PO}_4^{3-}$  ( $0.01$ – $2.4 \mu\text{mol L}^{-1}$ ). The regression scores of factor 2 were not significantly different between bloom and nonbloom samples ( $p = 0.693$ ) but were significantly different between the two populations ( $p = 0.001$ ). Population A was present in samples with relatively high  $\text{SiOH}_4$  concentrations ( $41$ – $81 \mu\text{mol L}^{-1}$ ) and low levels of solar irradiance ( $1,370$ – $4,449 \text{ W m}^{-2}$ ). In contrast, population B dominated under conditions of higher solar irradiance ( $4,371$ – $4,449 \text{ W m}^{-2}$ ) and lower  $\text{SiOH}_4$  concentrations ( $22$ – $42 \mu\text{mol L}^{-1}$ ) (Table 5).

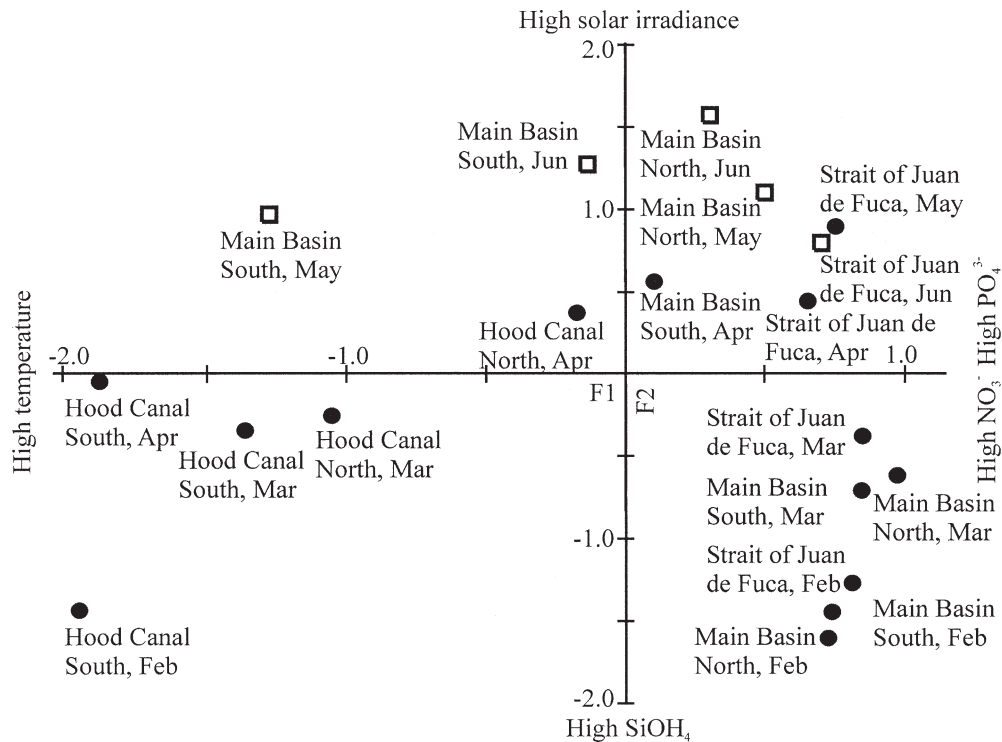


Fig. 6. Factor analysis of solar irradiance, temperature, salinity, and concentrations of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ , and  $\text{SiOH}_4$  for all water samples containing *D. brightwellii* in the spring of 2000. Factor 1 (F1) is positively correlated with high  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  concentrations and negatively correlated with high temperature. Factor 2 (F2) is positively correlated with high solar irradiance and negatively correlated with high  $\text{SiOH}_4$  concentrations. Filled circles and open squares represent water samples containing populations A and B of *D. brightwellii*, respectively.

## Discussion

*D. brightwellii* displayed multiple peaks in abundance, or blooms, during the spring growth season in hydrographically distinct basins of the Puget Sound and Strait of Juan de Fuca estuaries. In the slowly flushing Hood Canal basin inside Puget Sound, *D. brightwellii* bloomed in April. By June, cell abundance dropped to below detection, presumably due to water-column stratification and nutrient depletion (Newton et al. 2002). Both the Main Basin of Puget Sound and the Strait of Juan de Fuca are well-mixed estuaries (Ebbesmeyer and Barnes 1980), and blooms there occurred from March until June. Environmental conditions in both Puget Sound and the Strait of Juan de Fuca were variable over space and time and could not be used to predict bloom occurrence.

*Successive blooms comprised of genetically distinct populations*—There were no genetic differences among the three blooms that occurred in March and April of 2000 in different locations of Puget Sound and the Strait of Juan de Fuca. These blooms of greater than 1,000 cells  $\text{L}^{-1}$  were genetically indistinguishable from nonbloom samples that contained less than 1,000 cells  $\text{L}^{-1}$  sampled between February and April from the same region. This indicates that there was a single population of cells, defined as population A, that was present throughout the two estuaries early in the season. The fourth and last bloom

of the sampling period was composed of cells that were genetically distinct from the cells that bloomed earlier in the spring. This population was defined as population B and it was identified in all analyzed water samples collected in May and June. Blooms of both populations were relatively restricted in geographic extent. For example, population A bloomed in April in northern but not southern Hood Canal. In June, population B bloomed in the southern but not northern Main Basin. The limited extent of blooms in this hydrographically dynamic region suggests a close coupling of growth rate with local environmental conditions.

Population A could not be distinguished from the Puget Sound population that was sampled multiple times in both the spring and fall of the previous 2.5 yr (Rynewson and Armbrust 2004, 2005), suggesting that diatom populations can be long lived. Population B, however, was genetically differentiated from all previously identified populations. A total of four different *D. brightwellii* populations have been detected within the Puget Sound region on scales of 100s of km, suggesting that, on geographic scales of 1,000s of km, a broader diversity of populations exists and contributes to the bloom dynamics of this species.

*Sexual and asexual reproduction influence D. brightwellii population structure*—The populations sampled in this study were characterized by high levels of clonal diversity,

Table 5. Average and standard deviation of physical and chemical parameters throughout Puget Sound when populations A and B were present in spring of 2000.

Population (No. water samples)	$\text{NO}_3^-$ ( $\mu\text{mol L}^{-1}$ )	$\text{NO}_2^-$ ( $\mu\text{mol L}^{-1}$ )	$\text{NH}_4^+$ ( $\mu\text{mol L}^{-1}$ )	$\text{PO}_4^{3-}$ ( $\mu\text{mol L}^{-1}$ )	$\text{SiOH}_4$ ( $\mu\text{mol L}^{-1}$ )	Solar exposure ( $\text{W m}^{-2}$ $\text{day}^{-1}$ )	Temperature ( $^{\circ}\text{C}$ )	Salinity
Early ( $n=14$ )	$16.06 \pm 11.55$	$0.17 \pm 0.13$	$0.39 \pm 0.49$	$1.54 \pm 0.80$	$56.25 \pm 12.67$	$2,689 \pm 1175$	$9.06 \pm 1.57$	$27.72 \pm 3.64$
Late ( $n=5$ )	$11.83 \pm 7.40$	$0.28 \pm 0.13$	$1.29 \pm 0.81$	$1.31 \pm 0.56$	$33.62 \pm 8.14$	$4,402 \pm 42.72$	$10.51 \pm 0.91$	$29.04 \pm 0.98$

even during blooms when cell numbers exceeded 1,000 cells  $\text{L}^{-1}$ . The extent of clonal diversity is influenced by life-history traits, such as the occurrence and frequency of asexual and sexual reproduction and levels of interbreeding (Halkett et al. 2005). Planktonic diatoms inhabit a vast habitat and are constantly moving with currents, making examination of life-history traits in field populations challenging. Certain aspects of diatom life history, however, can be inferred from genetic markers. For example, asexual division produces genetically identical cells, or clonal lineages, that can be detected using microsatellite markers (Ryneckson and Armbrust 2005). In this study, the resampling of clonal lineages over several months and across nearly 200 km indicates that asexual division plays a role in generating multiple copies of a given clonal lineage that can then be physically dispersed throughout the range of the population.

The genetic recombination that occurs during sexual reproduction is an important mechanism for generating diversity. Based on high levels of clonal diversity and heterozygote deficiencies, it has been hypothesized that sexual reproduction occurred in a population identified inside Puget Sound (indistinguishable from population A identified in this study; Ryneckson and Armbrust 2005). The same genetic characteristics were present in population B, suggesting that sexual reproduction occurred and generated genotypic variation in this population as well. In some organisms, heterozygote deficiencies are observed across all loci examined and result from selfing during sexual reproduction (Viard et al. 1996). Although selfing may occur in *D. brightwellii*, it is an unlikely cause of heterozygote deficiencies because deficiencies were observed in only some of the loci analyzed. Null alleles are a more likely cause of the heterozygote deficiencies in two of the loci. Recent evidence that sexual reproduction is also a diversifying force in the pennate diatom *Pseudo-nitzschia pungens* (Evans et al. 2005) suggests that sex plays a significant role in generating variation across divergent taxa of diatoms. Currently, estimates of the frequency of sexual reproduction in field populations of diatoms range from once every year to once every 40 yr (Round et al. 1990). A future challenge will be to develop analytical methods to infer not only the signature of past sexual reproduction events but also their frequency from genetic markers.

The two populations were characterized by different valve diameters, a morphological characteristic used to infer the occurrence of sexual reproduction. In diatoms, asexual divisions result in differently sized daughter cells, one of which is smaller than the parent cell. As a result, the

average diameter of cells in a population becomes progressively smaller during periods of asexual division. Once cells reach a relatively small size, they can respond to environmental cues, undergo sexual reproduction, and restore valve diameter (Round et al. 1990). In culture, *D. brightwellii* cell diameters can range from 10 to 100  $\mu\text{m}$  (reviewed in Paasche 1973), a range similar to that found between populations A and B. One explanation for the progression from small valve diameters in early spring to large valve diameters in late spring is that population A underwent sexual reproduction and subsequent cell-size enlargement. However, the two populations had significantly different genetic compositions, indicating that a sexual reproduction event in population A did not give rise to population B. The process of sexual reproduction acts to recombine the existing genes in a population but generally does not alter its genetic composition. Furthermore, genetic characteristics, such as the hypothesized presence of a flanking mutation at *Dbr4* in nearly all cells from population B, and high  $F_{ST}$  values suggest greatly reduced levels of interbreeding and genetic exchange between the two populations (Hartl and Clark 1997). A more likely explanation for the differences in cell diameter is that populations A and B were at different stages of cell-size reduction. Whether populations A and B can obtain similar maximal cell diameters after sexual reproduction is unknown.

Although there was no evidence of sexual reproduction between populations during the sampling period, we looked for evidence of whether populations had the potential to interbreed by analyzing ribosomal DNA sequences. There were no genetic differences among isolates at the two most conserved regions analyzed (5.8S and 18S rDNA). Recent studies of cryptic species in the diatom genus *Skeletonema* showed that isolates from within a single species have up to 2 bp differences in the 18S rDNA sequence (Sarno et al. 2005). Approximately 1% sequence difference was detected between isolates from the two populations at the more variable regions (ITSI and ITSII). In the diatom *Sellaphora pupula*, isolates with 0.2% difference in 18S rDNA sequence and up to 7.3% difference in ITS sequence were able to mate and produce viable offspring (Behnke et al. 2004). Similarly, up to 10% sequence variation in the ITS regions of interbreeding individuals have been identified in volvocaceans (Coleman et al. 1994). Thus, a 1% sequence variation in the ITS region and identical 18S rDNA sequences suggest that interbreeding could occur between individuals from the two populations and that both populations represent a single species.

*Mechanisms driving population succession*—An abrupt transition in the genetic composition of *D. brightwellii* occurred within a 2-week window between April and May. The succession of genetically distinct populations involved three components: (1) the retention of population A within Puget Sound for at least 3 months prior to April, (2) the rapid loss of population A from the Main Basin between April and May, and (3) the rapid appearance and retention of population B in the Main Basin in May. Population A was retained within Puget Sound for 3 months and was also identified repeatedly over a 2.5-yr period. It has been hypothesized that recirculation of seaward flowing water in Puget Sound (Ebbesmeyer and Barnes 1980) allows for long-term retention of this population within the Sound (Rynearson and Armbrust 2004). In Hood Canal, population A may have been retained through May because all cells had small valve diameters characteristic of population A. Because these cells were in poor physiological condition and did not survive isolation into the laboratory, their genetic composition could not be determined. In the Main Basin, succession of populations occurred in May following the rapid loss of population A and appearance of population B. Although flushing of surface water is rapid in the Main Basin (Ebbesmeyer and Barnes 1980), valve-diameter measurements indicated that population A did not fully flush from the Main Basin: in May and June, about 1% of cells sampled from the Main Basin had small diameters characteristic of population A. The retention of small-diameter cells suggests that processes in addition to circulation led to the loss of population A from the Main Basin. The appearance of population B could be explained by circulation if Strait of Juan de Fuca water entering the Sound between April and May contained cells from population B. We looked for evidence of large valve diameters characteristic of cells from population B in the Strait of Juan de Fuca in early spring. In March, 6% of cells sampled from the Strait of Juan de Fuca had large valve diameters, whereas in Puget Sound, no cells with large valves were sampled. One month later, about 1% of the cells sampled within Puget Sound had large valve diameters, suggesting that cells from population B had begun to flush into the Main Basin of Puget Sound. A second possible explanation for the rapid appearance of population B is that cells from population B were inside Puget Sound prior to April but were not identified because concentrations were below detection.

The transition between populations was associated with changes in exposure to solar irradiance and concentrations of dissolved silicic acid. Exposure to solar irradiance is hypothesized to be a potent selective factor in field populations because light intensity significantly affects growth rates of genetically distinct clonal lineages in the laboratory (Gallagher 1982; Rynearson and Armbrust 2000, 2004). The magnitude of growth-rate variation among isolates from genetically distinct populations indicates that, in field populations, genetic composition could change dramatically on the order of weeks (Rynearson and Armbrust 2004), as was observed in this study. It is unknown how silicic acid might influence growth rates of genetically distinct clonal lineages. However, the concen-

tration of dissolved silicic acid has been hypothesized to be an important agent of selection among different species of diatoms (Rousseau et al. 2002) and, in *D. brightwellii*, appears to extend to regulating the dynamics of population succession. Succession of populations could thus have occurred if growth rates in population A decreased and growth rates of population B increased in response to high light intensities and low dissolved silicic acid concentrations.

We hypothesize that the dynamics of bloom formation and population succession result from a combination of recirculation, which acts to retain seeds of both populations inside Puget Sound and changing environmental conditions, which may select for individual populations. This combination of processes could explain the succession of populations during the spring growth season. Population A was repeatedly observed over 2.5 yr in both spring and fall (Rynearson and Armbrust 2004), suggesting the presence of an annual pattern of succession. Because no environmental data are available for samples collected in the fall, it is unknown whether the factors associated with succession in the spring extend to the remainder of the year. Factors, such as the germination of resting spores from genetically different populations, grazing, and parasitism, may influence population structure in *D. brightwellii* and in other diatom species and await future investigation.

*Biogeochemical and ecological implications of genetically distinct blooms*—The presence of two populations within a relatively restricted region that are composed of cells with dramatically different sizes suggests that genetically distinct populations may play different ecological and biogeochemical roles in aquatic habitats. The presence of differently sized populations at different times during the spring growth season could have consequences for carbon flux, silica cycling, and predation. For example, cells of different sizes have different biovolumes; large cells from population B have an order of magnitude greater biovolume and presumably a greater carbon content per cell than small cells from population A. Silica flux out of surface waters could be affected by cell size if differences in silica content differentially affect sinking rates. Finally, cells of different sizes may be susceptible to grazing by predators with different prey-size spectrums (e.g., Katechakis et al. 2004), and thus each population may have a different impact on food-web structure.

The succession of genetically distinct populations over multiple blooms and under distinct environmental conditions suggests a close coupling of environment and genetics that may be used to better explain, and perhaps predict, blooms of individual species, particularly for invasive and harmful algal-bloom species. Analysis of environmental and genetic variation over space and time may lead to improved prediction of phytoplankton responses to phenomena such as nutrient pulses (e.g., Karl et al. 2001), eutrophication (e.g., Beman et al. 2005), and climate change. For example, climate change may significantly change the local environments of phytoplankton populations through altered surface currents, upwelling, and

nutrient availability (e.g., Schmittner 2005). These changes may alter the type and strength of selection on locally adapted phytoplankton populations, as observed in terrestrial plants (Etterson 2004), changing bloom dynamics and their ecological and biogeochemical effects.

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