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Red Waters of *Myrionecta rubra* are Biogeochemical Hotspots for the Columbia River Estuary with Impacts on Primary/Secondary Productions and Nutrient Cycles

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Abstract The localized impact of blooms of the mixotrophic ciliate *Myrionecta rubra* in the Columbia River estuary during 2007–2010 was evaluated with biogeochemical, light microscopy, physiological, and molecular data. *M. rubra* affected surrounding estuarine nutrient cycles, as indicated by high and low concentrations of organic nutrients and inorganic nitrogen, respectively, associated with red waters. *M. rubra* blooms also altered the energy transfer pattern in patches of the estuarine water that contain the ciliate by creating areas characterized by high primary production and elevated levels of fresh autochthonous particulate organic matter, therefore shifting the trophic status in emergent red water areas of the estuary from net heterotrophy towards autotrophy. The pelagic estuarine bacterial community structure was unaffected by *M. rubra* abundance, but red waters of the ciliate do offer a possible link between autotrophic and heterotrophic processes since they were associated with elevated dissolved organic matter and showed a tendency for enhanced microbial secondary production. Taken together, these findings suggest that *M. rubra* red waters are biogeochemical hotspots of the Columbia River estuary.

Keywords *Myrionecta rubra* · *Mesodinium rubrum* · Red waters · Biogeochemical cycles · Columbia River estuary

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Introduction

The planktonic mixotrophic ciliate *Myrionecta rubra* (Jankowski), formerly *Mesodinium rubrum* (Lohmann), is distributed throughout the globe in marine and brackish waters, where it is known to generate non-toxic red tides (referred henceforth as red waters) in estuaries, fjords, and upwelling areas of the coastal ocean (Lindholm 1985). The intense red color of the blooms is the result of dense surface or subsurface aggregations (>10^4 cells mL^{-1}, Taylor et al. 1971) of *M. rubra* cells which are highly motile, phototactic and

contain several phycoerythrin-rich chloroplasts of cryptophyte algal origin (Smith and Barber 1979; Lindholm 1985; Dale 1987; Fenchel and Hansen 2006; Jiang 2011). Ciliate motility coupled with estuarine hydrography also leads to a horizontally patchy distribution of the red waters, with some observations >340 km^2 (Ryther 1967). Uncertainties remain concerning the exact conditions triggering the formation of the red water patches because irradiance alone does not seem to drive diel migration (Crawford and Lindholm 1997; Passow 1991).

In the Columbia River estuary, *M. rubra* blooms occur annually and last for several months during late summer through early fall (Herfort et al. 2011a). Recently, we determined that the Columbia River estuary bloom-forming *M. rubra* likely has an oceanic origin, and based on the nucleotide sequence analysis of a discriminating genetic marker (the internal transcribed spacer, ITS, sequence of *M. rubra* rDNA), this organism is one of at least five *M. rubra* haplotypes in the coastal waters of the Washington and Oregon coasts (Herfort et al. 2011a, c). Each year, the estuarine bloom is formed by a single *M. rubra* haplotype (haplotype B) population, which shows a strong specificity for chloroplasts originating from the cryptophyte *Teleaulax amphioxeia* (Herfort et al. 2011a). The bloom-forming *M. rubra* haplotype B first colonizes Ilwaco harbor located on the seaward-end of Baker Bay (Fig. 1), a shallow embayment near the river mouth, before establishing itself in the entire lower estuary (Herfort et al. 2011c).

The Columbia River estuary exhibits a complex pattern of chlorophyll distribution that is caused by relatively short water residence times, tidal mixing, seasonal variation of the source of allochthonous organic matter, and residence by *M. rubra* (Haertel et al. 1969; Roegner et al. 2010). Estuarine phytoplankton viability and primary productivity are thought to be low in the system (Haertel et al. 1969; Lara-Lara et al. 1990; Small et al. 1990), while allochthonous sources can be significant (Haertel et al. 1969; Sullivan et al. 2001; Roegner et al. 2010). In the spring, chlorophyll patterns are dominated by input of riverine diatoms, which can achieve high concentrations in the Columbia River (Small et al. 1990; Sullivan et al. 2001). However, most freshwater phytoplankton cells encountering the low salinity boundary (1–5) in the estuary tend to lyse due to osmotic stress (Lara-Lara et al. 1990). Short flushing times (0.5–5 days; Neal 1972) prevent adaptation to brackish conditions by non-motile organisms, and the remaining few viable phytoplankton cells do not achieve high primary productivity because of the light-limiting turbidity (Haertel et al. 1969; Frey et al. 1984; Lara-Lara et al. 1990; Simenstad et al. 1990). During the upwelling season, photosynthetic organisms are often

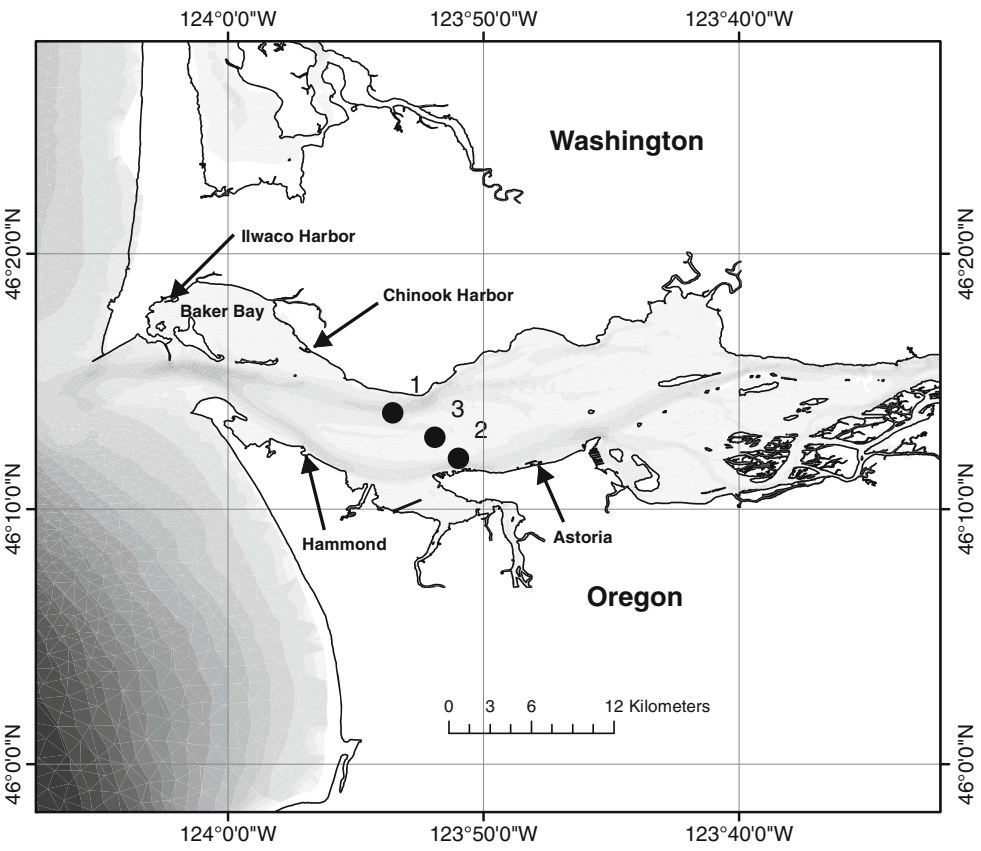

Fig. 1 Map of the Columbia River estuary showing the locations of water collection during *M. rubra* bloom periods in the north channel (1) for the duration of the time series conducted in 2007, 2009, and 2010; in and out of a red water patch in the south channel (2) and between the two channels (3) in 2008; in the south channel at Hammond or Astoria in 2010; and in Baker Bay at Ilwaco and Chinook harbors in 2010. *Shading* depicts bathymetry and highlights the estuary north and south channels

ocean-derived phytoplankton advected into the system with high salinity water (Haertel et al. 1969; Roegner et al. 2010; Herfort et al. 2011b). Although the fate of the ocean production in the estuary is unknown, the phytoplankton are also likely adversely affected by hypoosmotic stress. In contrast to most North American estuaries, past research has concluded that the Columbia River estuary is a detritus-based ecosystem with a thriving detrital food chain (Frey et al. 1984; Lara-Lara et al. 1990; Simenstad et al. 1990; Small et al. 1990).

Dense blooms of *M. rubra* likely have substantial biogeochemical impacts on the Columbia River estuary given that in other systems they exhibit very high photosynthetic rates. In fact, the highest microorganism primary production rates and chlorophyll *a* (chl *a*) concentrations ever recorded in aquatic systems were measured in a *M. rubra* bloom (Smith and Barber 1979). *M. rubra* blooms have caused oxygen supersaturation in the Southampton, Test and Columbia River estuaries as well as in the Baltic Sea (Soulsby et al. 1984; Lindholm 1986; Crawford et al. 1997; Roegner et al. 2011). *M. rubra* has the ability to assimilate ammonium, nitrate, amino acids, dissolved organic carbon and nitrogen (Smith and Barber 1979; Wilkerson and Grunseich 1990; Crawford et al. 1997) and to excrete dissolved organic matter (Smith and Barber 1979). Crawford and co-workers (1997) have even suggested that the peaks in bacterioplankton abundances observed after *M. rubra* blooms in the Southampton estuary were linked to the production of dissolved organic carbon (DOC) by *M. rubra*.

Thus, *M. rubra* red waters have the potential to affect several different components of biogeochemical cycles, and may challenge the traditional view of an allochthonously derived detritus-based Columbia River estuary. Consequently, three main questions arise:

1 What is the relationship between *M. rubra* cells and Columbia River estuarine biogeochemical cycles in red water patches?
2 Do *M. rubra* cells shift the trophic status of the Columbia River estuary from heterotrophy towards autotrophy in these red water patches?
3 If so, does this lead to a change in bacterial community structure and heterotrophic microbial activity in these red waters?

To address these questions, we enumerated *M. rubra* and diatom cells, measured concentrations of key photosynthetic pigments, of nutrients and of particulate and dissolved organic, and determined bacterial community composition and rates of microbial secondary production for water collected in the Columbia River estuary (main channels and Baker Bay) that were characterized by various levels of ciliate abundance during the *M. rubra* bloom periods of four consecutive years (2007 to 2010).

Materials and Methods

Study Area

The Columbia River estuary is located on the Pacific Northwest coast of the United States, between the states of Washington and Oregon (Fig. 1). Its main water source, the Columbia River, is the third largest river in the United States and Canada in freshwater discharge (2×10^{11} m^3) and supplies large inputs of particulate matter to the estuary. The basin of the estuary has two main channels and a few wide-mouthed lateral bays (Fig. 1). The south channel is dredged to enable shipping activities to Portland (Oregon), while the north channel is dredged moderately and shoals near the freshwater–brackish water interface. Baker Bay, which is located on the most seaward, north side of the Columbia River estuary, is typically less than 1.5 m depth, except for the dredged channels that enable access to its two small harbors: Ilwaco (river mile 3) and Chinook (river mile 6) (Fig. 1).

Sample Acquisition

All 51 water samples were collected during the *M. rubra* bloom periods in 2007–2010 when red waters were present throughout the lower Columbia River estuary and not just in Baker Bay. In late August 2007, an Eulerian sampling series was carried out in the Columbia River estuary north channel (Fig. 1) on board the R/V *Barnes* to collect six surface water samples (0–2 m) containing *M. rubra* cells in concentrations ranging from 0–1,600 cells mL^{-1}, using either a high volume, low pressure, air-driven (HVLP) pump or a bucket. In October 2008, two surface water samples were collected with a bucket in and out of a red water patch (3,000 and 600 *M. rubra* cells mL^{-1}, respectively) in the south channel and between the two channels (Fig. 1). In September 2009, an Eulerian sampling series was conducted using the HVLP pump on board the R/V *New Horizon* in the Columbia River estuary north channel (same location as 2007 time series; Fig. 1) to collect water samples during part of a tidal cycle. The set included three surface water samples (at 0.1–0.3 m depth) containing *M. rubra* cells in concentrations ranging from 2–350 cells mL^{-1}. In late August and September 2010, a total of 37 water samples, containing *M. rubra* in concentrations ranging from 0–1,500 cells mL^{-1}, were collected using either a bucket or a 1 L Van Dorn bottle (Lab Safety Supply, Janesville, WI, USA) at different depths (0–18.5 m) in the estuary south channel at Hammond or Astoria, and in the north channel at the same location as the time series of 2007 and 2009 (Fig. 1). In September 2010, three red water samples (0–3 m) were also obtained using a 1-L Van Dorn bottle in Baker Bay at Ilwaco and Chinook harbors (Fig. 1). Salinity (reported using the practical salinity scale) was

assessed in each sample using either the ship's (R/V *Barnes* and *New Horizon*) Seabird conductivity–temperature–depth (CTD) meter or a refractometer.

Bacterial 16S rRNA Gene Clone Libraries

Nucleic acids were extracted from samples collected in 2007 and 2008 as described in Herfort et al. (2011a). In short, 1 L of water was collected and immediately filtered through 0.2 μm-pore-size Sterivex filters (PES, ESTAR, Millipore) using a peristaltic pump. Samples were then fixed with 2 mL of RNAlater (Ambion) and stored at −80°C until processing. A phenol-based extraction was performed twice and extracts were combined. Bacterial 16S rRNA genes were amplified on a Bio-Rad DYAD PCR thermocycler using the universal bacterial primers 907f (5′-AAACTCAAAGGAATTGACGGG-3′) (Santegoeds et al. 1998) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane 1991) encompassing the variable regions V6–V9. The reaction mixture (25 μL) contained 2.5 μL 10× Taq polymerase buffer, 0.2 mM dNTP, 0.2 μM of each primer, 0.625 U Taq Polymerase, 3–15 ng of template DNA and water. Reactions were run according to the following PCR protocol: initial denaturation of 94°C for 4 min; 20 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, extension at 72°C for 90 s; and one final extension at 72°C for 7 min. For each sample, PCRs were run in triplicate and the products combined. These PCR products were then cloned and sequenced as described in Herfort et al. (2011a). Briefly, PCR products were ligated into a TOPO vector (pCR 2.1, Invitrogen) and used to transform One Shot Top 10 chemically competent *E. coli* cells (Invitrogen). For each sample, two 96-well microtiter plates (192 clones) were sequenced at the Genome Sequencing Center at Washington University (St. Louis, MO, USA) using the primer set mentioned above.

Sequence Analysis

For each 16S rDNA clone, the forward (907f) and reverse (1492r) sequence reads were combined by aligning them using the Smith–Waterman algorithm (Smith and Waterman 1981) implemented in the EMBOSS (European Molecular Biology Open Software Suite) package, using strict gap parameters (gap opening=50, gap extension=5.0). Clones were identified for which the Smith–Waterman alignment covered at least 300 bases with at least 97% identity, and a single combined sequence was generated in which positions of non-identity in the two reads were replaced with "N". BLAST was used to search the National Center for Biotechnology Information (NCBI) non-redundant nucleotide database for homologous sequences. Clones that had database hits with at least 300 bases aligned and with an expectation value≤1e-50 were retained for further analysis. Sequence data from this study have been deposited in GenBank database under accession numbers JF769888-JF770340.

Heterotrophic Microbial Production

Heterotrophic microbial production rates were determined for whole water samples collected in 2007 and 2009 by measuring the incorporation of L-[4,5-^{3}H] Leucine (20 nM final concentration at 69 Ci mmol^{-1}, Amersham) into the cold TCA insoluble fraction in four 1.7 mL sub-samples incubated on a rotator at 19°C (in situ temperature) for 1 h and processed following modifications of Smith and Azam (1992). Briefly, TCA-precipitated macromolecules were centrifuged at 13,000×*g* for 10 min, washed twice with cold 5% TCA, flooded with UltimaGoldXR scintillation cocktail and counted in a scintillation counter. Rates of leucine uptake were converted to rates of carbon production assuming a conversion factor of 3.09 kg C mol leu^{-1} (Kirchman et al. 1993).

Pigment Analysis by High Performance Liquid Chromatography

Suspended particulate matter was collected in 2007 and 2009 for pigment analysis by high-performance liquid chromatography (HPLC) by filtering water (100–300 mL) through GF/F filters (25 mm diameter, Whatman). The filters were then folded, wrapped in aluminum foil, and stored at −20°C in the dark until needed for photosynthetic pigment (chlorophylls and carotenoids) analysis by the HPLC method of Wright et al. (1991). Briefly, pigment samples were cold-extracted (−15°C) in polypropylene centrifuge tubes using a fixed volume of 90% acetone in water (*v*/*v*). Chromatographic separations were made using an Allsphere C8 reverse-phase column (25 cm×4.6 mm diameter, Grace) and diode array detection at 436 nm. Quantification was accomplished using response factors for authentic standards of chlorophyll *a* and fucoxanthin and the integrated peak area for each in the sample. No authentic standard for alloxanthin was available and so quantitative data for this carotenoid is reported in fucoxanthin equivalents.

Pigment Analysis by Fluorometry

Suspended particulate matter for pigment analysis by fluorometry was collected in 2008 and 2010 by filtering water (100–300 mL) through GF/F filters (25 mm diameter, Whatman). Chlorophyll *a* was cold-extracted (−15°C) in polypropylene centrifuge tubes using a fixed volume of 90% acetone in water (*v*/*v*). Samples were then examined with a Trilogy Laboratory Fluorometer (Turner Designs, Sunnyvale, USA) using the protocol described in Holm-Hansen et al. (1965) and a pure chlorophyll *a* standard

from *Anacystis nidulans* (Sigma) for purposes of instrument calibration.

Particulate Organic Carbon and Nitrogen

Suspended particulate matter for elemental (total organic carbon—POC, total nitrogen—PN) analysis was obtained for all samples (2007–2010 main channels and 2010 Baker Bay) by filtering 100–300 mL of water onto a pre-combusted (6–12 h at 500°C) GF/F filter (25 mm diameter, Whatman). Filters were folded, tightly wrapped in aluminum foil, and stored at −20°C until analysis. The POC and PN content of the suspended particulate matter on the acid-fumed filters (Hedges and Stern 1984) was determined using a Carlo Erba NA-1500 Elemental Analyzer (EA) system set-up and operated as described by Verardo et al. (1990).

Nutrients

For all samples (2007–2010 main channels and 2010 Baker Bay), water filtered through a GF/F filter (25 mm diameter, Whatman) was collected in acid-washed polyethylene vials (25 mL) and stored at −20°C until analysis. Concentrations for ammonium, nitate+nitrite, nitrite and ortho-phosphate were determined using an Astoria-Pacific continuous segmented flow analyzer set up and operated as described by Gordon et al. (1994) and Sakamoto et al. (1990).

Total Dissolved Nitrogen and Phosphorus

For all samples (2007–2010 main channels and 2010 Baker Bay), water (20 mL) filtered through a GF/F filter (25 mm diameter, Whatman) was collected into pre-conditioned 30 mL polypropylene bottles and stored at −20°C until analysis. Total dissolved nitrogen and phosphorus were measured simultaneously by Horn Point Laboratory analytical services using the method of Valderrama (1981). Concentrations of dissolved organic nitrogen and phosphorus (DOP and DON) were calculated from these values by subtracting concentrations of dissolved inorganic nitrogen (nitrite, nitrate, and ammonium) and phosphorus.

Dissolved Organic Carbon

For all samples (2007–2010 main channels and 2010 Baker Bay), water (20 mL) filtered through a GF/F filter (25 mm diameter, Whatman) was collected in polypropylene vials and stored at −20°C until analysis. The samples were analyzed by Horn Point Laboratory analytical services using a Shimadzu TOC-5000 total organic carbon analyzer set up and operated as described by Sugima and Suzuki (1988).

Light Microscopy

For cell counts, the 2007–2008 water samples (40 mL) were fixed with formaldehyde (final concentration, 4%) at room temperature for 1 h and stored at −20°C for a week until returning to the lab where they were placed at −80°C until analysis. The samples were then thawed and cells from a prescribed volume (25 mL) were settled overnight according to a published method (Utermöhl 1931, 1958). The 2009–2010 water samples (40 mL) were fixed with Lugol's Iodine (final concentration 1%) and stored at 4°C until analysis as described above. A minimum of 100 cells were counted per sample using an inverted microscope (Apotome, Zeiss or Leica, Bartel & Stout Inc.). The light microscopy approach employed to detect fixed *M. rubra* cells was previously validated in an earlier study (Herfort et al. 2011a) in which (1) *M. rubra* cells were still clearly visible when present, even though cells of this ciliate fixed with formaldehyde and stored frozen were often damaged, and (2) similar *M. rubra* cell counts were reached when comparing samples fixed with formaldehyde to those fixed with Lugol's Iodine. *M. rubra* abundance was determined for all samples, while that of diatoms was only established for 2007–2008 samples. *M. rubra* cell counts reported as zero indicated that the ciliate was not detectable in the 25 mL sample.

Statistical Analysis

Spearman's rank correlation analyses were performed using the plymouth routines in multivariate ecological research (PRIMER) software version 6 (PRIMER-E Ltd, UK). Patterns of bacterial diversity across the nine 2007–2008 surface samples were examined by UniFrac analysis (Lozupone and Knight 2005) of aligned bacterial 16S rRNA gene sequences using a Bonferroni correction and 100 permutations. These 2007–2008 samples were arranged in three sets—*M. rubra*-rich (red water, >1,000 cells mL^{-1}), containing lower levels of *M. rubra* (150–600 cells mL^{-1}) and no *M. rubra*—and UniFrac analysis was done between groups.

Results

Water samples collected in the estuary main channels and in Baker Bay (Ilwaco and Chinook harbors) during bloom periods of 2007–2010 were sorted into three sets based on their cell concentration: *M. rubra*-rich (red water) (1,000–3,500 cells mL^{-1}), containing some *M. rubra* (non-bloom water) (1–1,000 cells mL^{-1}) and no *M. rubra* (<1 cell mL^{-1}).

Microscopic Cell Counts

For water collected during the bloom periods of 2007–2008, the number of *M. rubra* and diatom cells in the three sets of

samples is reported as percentages in Table 1. Note that diatoms are the only phytoplankton reported in this study because they were the most abundant protist group in *M. rubra*-free waters (data not shown), and because historically this group has been shown to dominate the protist assemblage of the Columbia River estuary (Haertel et al.1969; Frey et al. 1984; Small et al. 1990). Diatom cells were always second in abundance to *M. rubra* in samples containing the ciliate (1–3,000 cells mL^{-1}) (data not shown). *M. rubra* cells were at least ten times more abundant than diatom cells in red waters, and they outnumbered diatoms by at least a factor of 2–4 in the non-red water samples containing fewer *M. rubra* cells (1–1,000 cells mL^{-1}) (Table 1).

Photosynthetic Pigment Analysis

Average chl *a* concentrations in the main channels of the estuary were extremely high (75.7 μg L^{-1}) in red waters, were ten-fold lower (7.7 μg L^{-1}) in non-bloom samples, and were very low (1.1 μg L^{-1}) in samples lacking *M. rubra* cells (Fig. 2b). Average concentrations for alloxanthin, a diagnostic pigment for the *M. rubra* cryptophyte chloroplast, and for fucoxanthin, a diagnostic pigment for diatoms, followed similar patterns to that of chl *a* (Table 1). The distributions of chl *a*, alloxanthin, and fucoxanthin in the estuary main channels were significantly correlated with *M. rubra* cell counts based on Spearman's rank correlation analysis ($p<0.001$; rho=0.812; $n=46$ for chl *a*; $p<0.01$; rho=0.964; $n=7$ for alloxanthin; $p<0.05$; rho=0.893; $n=7$ for fucoxanthin). Nonetheless, alloxanthin concentrations were on average 12 and 10 times greater than those of fucoxanthin in red waters and in samples with fewer *M. rubra* cells, respectively. Furthermore, the alloxanthin to chl *a* ratio was not only high in red waters (5.31) but was also ~7 times higher than the fucoxanthin to chl *a* ratio in both red waters and waters containing fewer cells of the ciliate (Table 1). High chl *a* concentrations (56.8 μg L^{-1}), comparable to those measured in the main channel's red water, were also found in the red water of Baker Bay (Fig. 2b).

Molecular Analysis of the 16S rRNA Gene of Bacteria

The relative abundance of bacterial 16S rRNA gene sequences of the 11 different bacterial groups found in our 2007 and 2008 surface water clone libraries are presented in Table 2. In general, Cyanobacteria, Actinobacteria, Bacteroidetes, α-, β-, and γ-Proteobacteria sequences dominated the clone libraries, but there was no clear distribution pattern associated with *M. rubra* abundances ($p>0.05$ for UniFrac significance test among the three sample sets sorted by *M. rubra* abundance—red waters, few *M. rubra* cells, and no *M. rubra*). Note that in contrast to Romalde et al. (1990) and based on classification obtained using the Ribosomal Database Project (http://rdp.cme.msu.edu/classifier/classifier.jsp), the Vibrionales were not associated with *M. rubra* cells in our dataset, and in fact represented only 1.4% of all our γ-Proteobacteria sequences (data not shown).

Heterotrophic Bacterial Production

The rates of L-[4,5-^{3}H] leucine incorporation (expressed as carbon production rates) in the three sets of samples sorted by *M. rubra* cell content for water collected in the estuary north channel during the bloom periods of 2007 and 2009 are presented in Fig. 2c. Heterotrophic microbial production was higher in red waters (1.25 μg CL^{-1} h^{-1}) and in water containing fewer *M. rubra* cells (1.00 μg CL^{-1} h^{-1}) than in the sample lacking *M. rubra* cells (0.40 μg CL^{-1} h^{-1}).

Dissolved Organic and Inorganic N and P

Concentrations of dissolved organic and inorganic nutrients as well as calculated N/P ratios (nitrate/Dissolved Inorganic

Table 1 Relative contribution of *Myrionecta rubra* and diatom cells detected by light microscopy as well as concentrations and ratios of their diagnostic pigments (alloxanthin and fucoxanthin, respectively) for samples collected in the Columbia River estuary main channels in red water (>1,000 *M. rubra* cells mL^{-1}), water containing some *M. rubra* (1–1,000 cells mL^{-1}) and water without *M. rubra* cells during the bloom periods of 2007 and 2008

		Red water	Some *M. rubra*	No *M. rubra*
Relative proportions of cell counts (%)	*M. rubra*	97 (4)	73 (3)	0
	Diatom	3 (4)	27 (3)	100
Photosynthetic pigments (μg L^{-1})	Alloxanthin	51 (21.2)	3 (0.7)	0.2
	Fucoxanthin	7 (4.8)	0.4 (0.1)	0.3
Photosynthetic pigment ratios	[Alloxanthin]/[Chlorophyll *a*]	5.31 (0.08)	0.41 (0.07)	0.03
	[Fucoxanthin]/[Chlorophyll *a*]	0.73 (0.03)	0.05 (0.02)	0.05

Note that pigments were not analyzed in 2008 and that diatoms are the only phytoplankton reported here because historically this group has been shown to dominate the protist assemblage of the Columbia River estuary (Haertel et al. 1969; Frey et al. 1984; Small et al. 1990). Standard errors are given in brackets

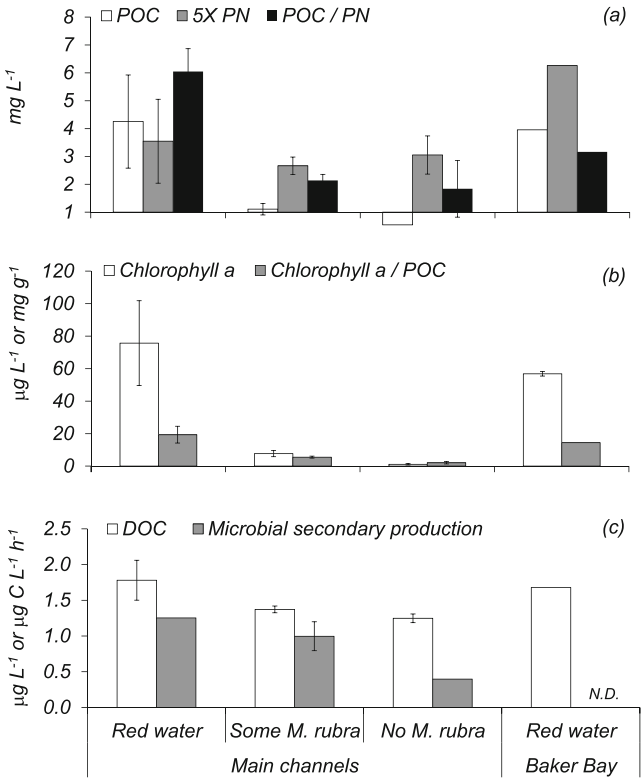

Fig. 2 Concentrations of particulate and dissolved organic carbon (POC (**a**) and DOC (**c**)), particulate nitrogen (PN) (**a**), chlorophyll *a* (**b**), as well as rates of microbial secondary production determined by leucine incorporation (**c**) for samples collected in the Columbia River estuary main channels in red water (>1,000 cells mL^{-1}), water containing some *M. rubra* (1–1,000 cells mL^{-1}) and water without *M. rubra* cells or for samples gathered in Baker Bay (Chinook and Ilwaco harbors) in red waters (1,000–3,500 cells mL^{-1}) during the bloom periods of 2007–2010. *Bars*=standard errors. *N.D.*=not determined

Phosphorus (DIP) and Dissolved Inorganic Nitrogen (ΣDIN)/DIP) for waters collected in the estuary main channels or in Baker Bay during the bloom periods in 2007–2010 are provided in Fig. 3a, b, c. Red waters in the estuary main channels contained higher DIP, DON, and DOP but lower ammonium and N/P ratios than waters containing fewer and no *M. rubra* cells. Red waters of Baker Bay differed from that of the main channels in that they contained higher ammonium, nitrate, and N/P ratios but lower DIP. The concentrations of DON and DOP in main channels and Baker Bay were correlated with *M. rubra* cell abundance (Spearman's rank correlation analysis: $p<0.01$; rho=0.471; $n=46$ for DON, and $p<0.001$; rho=0.542; $n=46$ for DOP) (Fig. 4b, c).

Particulate N

M. rubra abundances were not correlated with concentrations of particulate nitrogen (PN) in the estuary main channels, with averages for the three *M. rubra* sets ranging from 0.53 to0.71 mg L^{-1}. However, high PN concentrations were associated with the red waters of Baker Bay (1.25 mg L^{-1}) (Fig. 2a).

Particulate and Dissolved Organic Carbon

Concentrations of particulate and dissolved organic carbon (POC and DOC) were positively correlated with *M. rubra* abundances in the estuary main channels (Figs. 2a, c and 4a; Spearman's rank correlation analysis, $p<0.001$; rho=0.696; $n=45$ for POC and $p<0.01$; rho=0.430; $n=44$ for DOC), and the red waters of the main channels and of Baker Bay contained similarly high levels of POC (4.3 and 4.0 mg L^{-1}, respectively) and DOC (1.8 and 1.7 µg L^{-1}, respectively) (Figs. 2a, c and 4a).

The Relationship between *M. rubra* Abundance and the Salinity of Estuarine Water Masses

Although the 51 water samples of this study were collected over a wide range of salinity (1.5–32), elevated *M. rubra* abundances were mostly found in water with salinity below 15 (Fig. 4a–c). This restricted distribution is also apparent in the lack of correlation between *M. rubra* abundance and salinity (Spearman's rank correlation analysis, $p>0.05$; rho=−0.148; $n=49$) and in the fact that salinity values of red waters from main channels and Baker Bay were restricted between 3.6 and 10, while that of waters containing some *M. rubra* cells and those lacking cells span a larger range of salinity (1.5–32) (Fig. 4a–c).

Note that physical, biological, and chemical data of the 51 water samples used for calculating the averages presented on Figs. 2, 3, and 4 are provided in supplementary Table S1.

Discussion

M. rubra Blooms and Dissolved Nutrient Levels

Ammonium was negatively correlated with *M. rubra* abundance in the Columbia River estuary main channel (Spearman's rank correlation analysis, $p<0.01$; rho=−0.430; $n=48$), with lower concentrations detected in red waters (Fig. 3a). This is not surprising because ammonium has been shown to be the preferred nitrogen source for *M. rubra* in the Southampton estuary (Crawford et al. 1997), and high rates of inorganic N nutrient uptake (2.1–15.5 µg NL^{-1} h^{-1}) have been observed within *M. rubra* blooms (Smith and Barber 1979; Dugdale et al. 1987; Wilkerson and Grunseich 1990). The ammonium/nitrate ratios calculated for the estuary main channel samples were also negatively correlated with *M. rubra* abundance (Spearman's rank correlation analysis, $p<0.01$; rho=−0.398; $n=48$), with values in red waters

Table 2 Relative contribution, given as percent of total, of different bacteria 16S rRNA gene sequences to the total number of bacterial clones in libraries constructed from surface waters (0–2 m) collected in the Columbia River estuary main channels during the bloom periods of 2007 and 2008

Samples	Red water				Some *M. rubra*				No. *M. rubra*
	2	5	7	8	1	3	6	9	4
Actinobacterium	13	**43**	1	6	5	12	**29**	2	5
Bacteriodetes	**21**	14	0	**44**	1	**58**	25	**42**	**55**
Alpha proteobacteria	17	29	5	33	10	3	14	37	16
Beta proteobacteria	17	0	1	8	0	2	15	5	2
Gamma proteobacteria	4	14	**73**	8	**73**	12	10	12	11
Delta proteobacteria	8	0	0	0	0	2	0	0	0
Firmicutes	**21**	0	0	0	0	2	2	0	2
Plantomycetes	0	0	0	0	0	2	2	0	0
Verrucomicrobia	0	0	0	0	0	3	3	2	9
Acidobacteria	0	0	20	0	10	2	0	2	0
Fibrobacteres	0	0	0	0	0	3	0	0	0

Samples are organized according to their *M. rubra* content: red water (>1,000 cells mL^{-1}), water containing some *M. rubra* (150–600 cells mL^{-1}) and water without *M. rubra* cells. Samples numbers refer to the chronological order in which water was collected. The most abundant bacterial group for each sample is highlighted in bold. The data show no clear pattern of bacterial community structure associated with *M. rubra* abundance (confirmed by UniFrac analysis, $p>0.05$)

(0.44 SE±0.22) and waters containing fewer *M. rubra* cells (0.43 SE±0.06) being twice as low as those found in waters with no *M. rubra* (1.16 SE±0.39) (data not shown). This finding supports the idea of a preferential removal of ammonium over nitrate by *M. rubra* in the Columbia River estuary main channels during the bloom period.

Nonetheless, *M. rubra* is also able to use nitrate (Wilkerson and Grunseich 1990), as observed in the Southampton estuary, where the ammonium/nitrate uptake ratio tends to decrease as the bloom ages, resulting from enhanced nitrate uptake (Crawford et al. 1997). Crawford et al. 1997 argue that the 1985 and 1986 *M. rubra* blooms in this British estuary were not nitrogen limited across most of the estuary based on relatively elevated depth-integrated concentrations of nitrate and ammonium during times of peak *M. rubra* abundance. However, in the Columbia River estuary, the N/P ratio (nitrate/DIP) within red waters was low in the main channels (6.6; Fig. 3c), providing evidence that nitrogen might be the limiting nutrient. In contrast, samples containing fewer or no *M. rubra* cells had a nitrate/DIP ratio that was similar or above the Redfield value of 16 (Fig. 3c). When concentrations of all DIN (ΣDIN) were used to calculate the N/P ratio, this difference was even more apparent, with ratios above 25 for waters containing some or no *M. rubra* and ratios below 16 for red waters (Fig. 3c). It is important to note that putative N-limitation in the bloom as indicated by low N/P ratios was not a sporadic event since the same pattern was apparent each year of the study in both north and south channels of the estuary. This indicates that the surface red waters of the lower Columbia River estuary main channels might be nitrogen-limited but the rapid jumping behavior of *M. rubra* might, however, enhance nutrient uptake beyond the limitation of molecular diffusion in red water patches (Fenchel and Hansen 2006; Jiang 2011) and also allow cells to move rapidly into more nutrient-rich waters.

Ammonium concentration in Baker Bay red waters (6.6 μM) was higher than in the estuary main channel waters (Fig. 3a). While the average ammonium/nitrate ratio in the red waters of Baker Bay (0.89 SE±0.34) was still below 1, it was higher than that calculated for the red waters of the main channels (data not shown). This indicates that during the bloom period while ammonium is still the preferred nitrogen source, the contribution of nitrate is likely higher in red waters of Baker Bay than the main channels. Since the same genetic population of *M. rubra* (haplotype B) is present in the two areas (Herfort et al. 2011a), it is likely that environmental conditions rather than cell specificity dictate *M. rubra* nitrogen preference. In accordance with this observation, during upwelling off the Peruvian coast, preference between ammonium and nitrate could not be discerned under light-saturated conditions, likely because of the high in situ nitrate concentrations, although dark ammonium uptake by *M. rubra* was prevalent (Wilkerson and Grunseich 1990). Furthermore, unlike the main channels of the estuary, red waters in Baker Bay (Chinook and Ilwaco harbor) did not appear to be N-limited since N/P ratios (nitrate/DIP and ΣDIN/DIP) were above the Redfield value (16), and concentrations of both ammonium and nitrate were much higher than those measured anywhere in the estuary main channels (Fig. 3c). This discrepancy in N/P ratios between red water

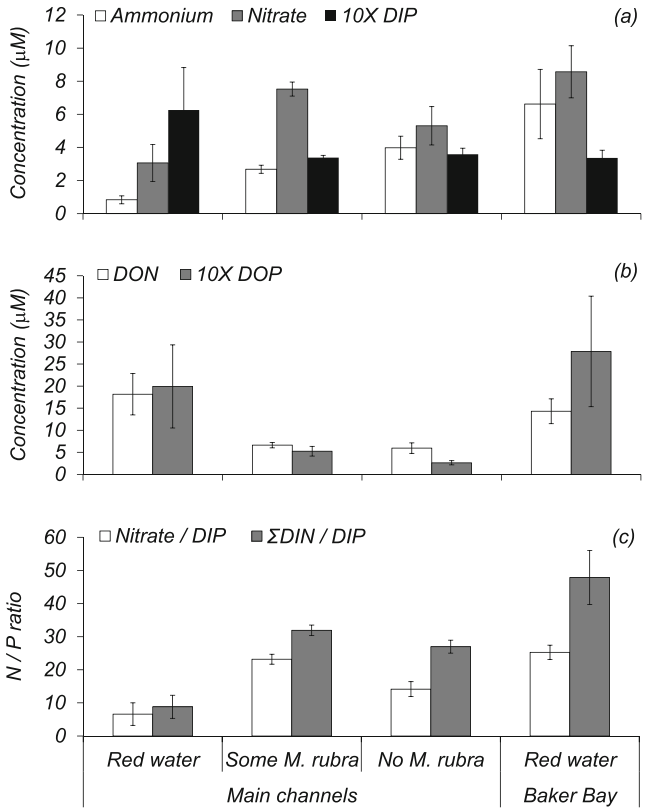

Fig. 3 Concentrations of ammonium (**a**), nitrate (**a**), dissolved inorganic phosphorus (DIP) (**a**), dissolved organic nitrogen (DON) (**b**) and phosphorus (DOP) (**b**) as well as N/P ratios (**c**) (nitrate/DIP and dissolved inorganic nitrogen (ΣDIN)/DIP) for samples collected in the Columbia River estuary main channels in red water (>1,000 cells mL^{-1}), water containing some *M. rubra* (1–1,000 cells mL^{-1}) and water without *M. rubra* cells or for samples gathered in Baker Bay (Chinook and Ilwaco harbors) in red waters (1,000–3,500 cells mL^{-1}) during the bloom periods of 2007–2010. The data in the main channels shows higher DIP, DON, and DOP, but lower ammonium, nitrate, and N/P ratios in red waters. Red waters of Baker Bay were characterized by higher ammonium, nitrate, and N/P ratios but lower DIP than those of the main channels. *Bars*= standard errors

masses in the two areas containing similarly high *M. rubra* abundances (1,000–3,500 cells mL^{-1}) is particularly interesting because this shows that putative N-limitation in red waters is strongly dependent on environmental nutrient levels.

Throughout the lower Columbia River estuary (main channels and Baker Bay), *M. rubra* cell abundance was correlated with DON and DOP concentrations (Fig. 4b, c), and these organic nutrient sources may be important for fueling *M. rubra* blooms once inorganic nutrient sources have been depleted. The correlation between DON and *M. rubra* abundance is particularly interesting because DON (but not DOP) was also negatively correlated with salinity (Spearman's rank correlation analysis, $p<0.01$; rho=−0.392; $n=44$), while no correlation existed between *M. rubra* abundance and salinity (Fig. 4b, c). In fact, elevated ciliate cell numbers seem to be restricted to water masses with salinity below 15 (Roegner et al., 2010; Fig. 4a–c). This suggests that although variations in DON may arise from the different water sources, the presence of high *M. rubra* numbers in red water alone also has an important impact. Nevertheless, the occurrence of high DON concentrations alongside low nitrate concentrations (Fig. 3b) suggests that inorganic nutrients are the preferred nitrogen source in main channel red waters.

M. rubra cells shift the energy transfer pattern towards autotrophy in red water patches

In red water patches, *M. rubra* cells also have an impact on the source of particulate organic matter and the local trophic status. Indeed, it is generally thought that fast flushing times and high turbidity reduce the viability of the phytoplankton standing stock in the Columbia River estuary and limit primary productivity (Frey et al. 1984). This leads to a heterotrophic microbe-centric ecosystem where most organic matter is typically present in the form of detrital organic matter delivered from the adjacent river and ocean (Frey et al. 1984; Lara-Lara et al. 1990; Small et al. 1990; Sullivan et al. 2001; Roegner et al. 2010). In contrast, during *M. rubra* blooms, this mixotrophic ciliate clearly dominated the estuary main channel phytoplankton assemblage whenever present (Table 1). In addition, concentrations and pigment ratio of *M. rubra* diagnostic cryptophyte chloroplast pigment, alloxanthin (Goodwin 1971), were high (Table 1). Diatoms were at least ten times less abundant than *M. rubra* cells within the red water patches and concentrations of fucoxanthin, a diagnostic pigment for diatoms, were consistently low (Table 1). Note that although fucoxanthin is present in a variety of algal taxa, this pigment is most commonly associated with diatoms and is thus frequently used as a chemotaxonomic marker for diatoms (Goodwin 1971; Sullivan et al. 2001). In waters containing fewer *M. rubra* cells, diatoms were still outnumbered by the ciliate by a factor of 2–4 and fucoxanthin concentrations remained low (Table 1). Furthermore, the alloxanthin to chl *a* ratio was ~7 times higher than the fucoxanthin to chl *a* ratio in both red waters and waters containing fewer cells of the ciliate (Table 1). Notably, the high alloxanthin to chl *a* ratio (5.3) detected in red water patches is much higher than that commonly measured for cryptophytes, even when compared with the relatively high Ross Sea value of 1.04±0.22 reported by DiTullio et al. (2003). These high red water alloxanthin to chl *a* ratios (5.3) are also much higher than those (<0.25) obtained during a *M. rubra* bloom in the Bedford Basin, a marine coastal inlet off the coast of Nova Scotia (Canada) (Kyewalyanga et al. 2002). Given that the Columbia River estuary is an extremely turbid environment which typically causes phytoplankton primary production to be light-limited (Haertel et al. 1969; Frey et al. 1984; Lara-Lara et al. 1990;

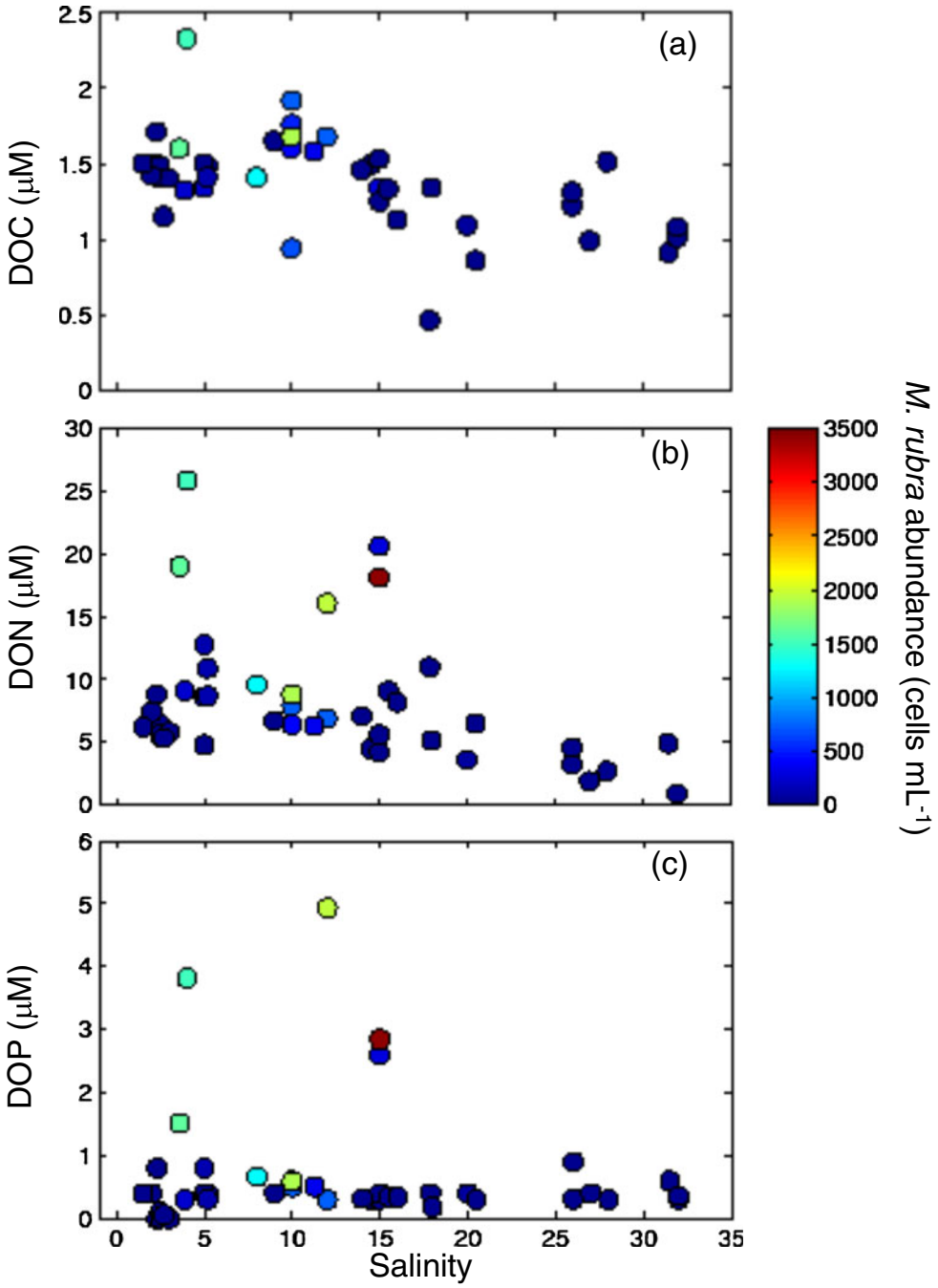

Fig. 4 Plots of dissolved organic carbon (DOC) (**a**), nitrogen (DON) (**b**), and phosphorus (DOP) (**c**) vs. salinity values with *M. rubra* cell abundances as colored dots for water collected in the Columbia River estuary (main channels and Baker Bay) during the *M. rubra* bloom periods of 2007–2010. Data show that while elevated *M. rubra* abundances were restricted to water with salinity values below 15, they were associated with increased concentrations of dissolved organic matter (confirmed by Spearman's rank correlation analysis, $p<0.05$)

Simenstad et al. 1990), the high red water alloxanthin to chl *a* ratios detected in this system likely indicate *M. rubra* cells adaptation to the extreme low-light conditions found in this estuary. Together, our pigment and cell count data show that during *M. rubra* blooms, the proliferation of phytoplankton was almost solely carried out by *M. rubra* in waters containing cells of the ciliate, suggesting that ciliate bloom events have the potential to dramatically alter patterns of energy transfer within the estuary (especially at local scales in red water).

Similar to observations from studies of other estuaries (Smith and Barber 1979; Taylor 1982; Lindholm 1985; Crawford 1989), high photosynthetic rates were likely achieved by *M. rubra* in the Columbia River estuary main channels despite being found in a turbid, rapidly-flushed system. In order to estimate the chl *a* per *M. rubra* cell, and given that *M. rubra* represents more than 90% of the phytoplankton assemblage in the estuary main channel red waters (Table 1), we assumed that the majority of the chl *a* measured in the red water patches was associated with the

ciliate (which the high alloxanthin to chl *a* ratio indicates; Table 1). With a spherical shape and an average diameter of 37 and 40 μm (determined by microscopy for 2007 and 2010, respectively), the chl *a* to volume ratio was calculated to be 0.8−2.7 and 1.7 fg μm^3 for the estuary main channel red water patches in 2007 and 2010, respectively. This is in relatively good agreement with the 2.6 and 2.7 fg μm^3 measured for *M. rubra* isolates from two small estuaries in Falmouth (Massachusetts) (Stoecker et al. 1991) and blooms in the Southampton estuary (Crawford et al. 1997), and thus supports the idea that *M. rubra* cells account for most of the chl *a* in the Columbia River estuary red waters. Based on both chl *a* concentrations and taxon-specific pigment ratios normalized to chl *a*, the potential for photosynthesis was almost 70 times higher in red waters than in the typical surface estuary water for which the predominant phototrophs were diatoms (no *M. rubra*) (Table 1). For waters containing fewer *M. rubra* cells (non-bloom waters), this potential for photosynthesis was on average seven times higher than that of water lacking *M. rubra* (Table 1). These findings are in accordance with comparisons of *M. rubra*-specific and community photosynthetic rates conducted in Falmouth estuarine waters containing less than 1,000 *M. rubra* cells mL^{-1}, which also demonstrated important contributions of the ciliate to community primary production under non-bloom conditions (Stoecker et al. 1991).

Unlike diatoms, *M. rubra* is able to achieve high primary productivity in the turbid waters of the Columbia River estuary because the photosynthetic machinery of its cryptophyte chloroplasts is well adapted to dim light. Indeed, cryptophytes have a combination of photosynthetic pigments (chlorophyll *a* and *c*, phycobilins, and carotenoids) that facilitates the efficient absorption of the dim blue/green wavelengths (Bergman et al. 2004), which are typical of the environments where they are usually found (e.g., deep oligotrophic waters, coastal oceans, estuaries, and lakes) (Ilmavirta 1988; Klaveness 1988; Tamigneaux et al. 1995; Pinckney et al. 1998). In addition, whilst cryptophytes do not produce the photoprotective compounds necessary to shield them from the damaging effects of elevated photosynthetically active and ultraviolet radiations (Vernet et al. 1994) likely present where red waters form (i.e., very surface of the estuarine water column), *M. rubra* does possess mycosporine-like amino acids that can provide protection against UV radiation (Johnson et al. 2006).

During the 2007–2010 blooms, POC was higher when *M. rubra* was present than in water lacking the protist (Fig. 2a). Similar increases in particulate organic matter (POC or PN) have been reported in water containing *M. rubra* in Barrow (Alaska), Baja (California), and the coastal upwelling of Peru (Holm-Hansen et al. 1970; Packard et al. 1978; Wilkerson and Grunseich 1990). The chl *a*/POC ratio, which indicates the quality of particulate organic matter, varied between our estuary main channel samples, with values of 19.3 mg/g for red waters, 5.5 mg/g for water containing less *M. rubra* cells and 2.1 for waters without *M. rubra* (Fig. 2b). For healthy phytoplankton, this ratio ranges between 10 and 30 mg/g (Sullivan et al. 2001 and references therein). Given the results discussed above showing that most of the chl *a* in *M. rubra*-rich waters is stored in the ciliates (cell counts, pigment concentrations, but especially pigment ratios; Table 1), these high chl *a*/POC ratios suggest that the majority of POC in surface waters containing *M. rubra* cells is derived from the ciliates, and not from allochthonous input of river-borne (or oceanic) phytoplankton or soil organic matter (Simenstad et al. 1990). In support of this idea of an autochthonous source of organic matter from *M. rubra* blooms, it is noteworthy that DNA from dead *M. rubra* cells from red water bloom decay was detected in water samples collected 1 m above the bottom in the south channel of the estuary in late August 2007 (Herfort et al. 2011b).

All the aforementioned findings challenge the traditional view of a late summer/early fall allochthonous-derived detritus-based Columbia River estuary, since they demonstrate dominance of *M. rubra* in the phytoplankton assemblage. This has the potential to dramatically alter patterns of energy transfer within the Columbia River estuary by raising the autotrophic status of the estuary in waters where the ciliate is prevalent. Thus, in the areas of the estuary where the ciliate is abundant, *M. rubra* contributes autochthonous organic matter to supplement the large allochthonous inputs. *M. rubra* red waters can therefore be viewed as hotspots of primary productivity in the Columbia River estuary.

Links between Autotrophic and Heterotrophic Processes

This energy transfer shift towards autotrophy in areas where *M. rubra* cells are abundant takes place in an ecosystem typically regarded as detritus-driven and characterized by an active microbial community that can support up to 84% of the estuarine secondary production (Simenstad et al. 1990). The question arises as to what is the impact of this recurring hotspot of in situ primary productivity, and its associated autochthonous organic matter inputs, on the heterotrophic microbial production of the water in which *M. rubra* is prevalent, in terms of both rates and community structure. Based on bacterial 16S rRNA gene sequences sorted according to their *M. rubra* cell content (Table 2) and analyzed with UniFrac, the bacterial community structure present in the water samples obtained in 2007–2008 did not vary significantly with *M. rubra* cell abundance ($p>0.05$ for all).

In contrast, rates of heterotrophic microbial production assessed by leucine incorporation were slightly higher in red waters and in water with fewer *M. rubra* than in the water

sample devoid of *M. rubra* cells (Fig. 2c). It is important to note that, perhaps due in part to our small sample set (n=9), the ciliate's abundance was not statistically correlated with rates of microbial secondary production (Spearman's rank correlation analysis p>0.05; rho=0.283; n=9). Nonetheless, the low rate of heterotrophic microbial production measured here in the estuarine surface sample devoid of *M. rubra* cells is, in fact, in good agreement with other heterotrophic microbial production rates (≤0.41 μg CL^{-1} h^{-1}) obtained during the same cruise (August 2007) in nine low salinity (≤6.01) estuarine surface water samples characterized by similarly low chlorophyll fluorescence values (≤1.04 mg m^3), and therefore, likely also devoid of *M. rubra* cells (data not shown). Thus, taken together these data suggest a trend whereby heterotrophic microbial production rates are somehow increased by the presence of *M. rubra* cells. Elevated rates of leucine incorporation in waters containing *M. rubra* cells could be due to increased bacterial production and/or result from direct uptake of leucine by *M. rubra*. At this point, it is impossible to determine which scenario is correct because Smith and Barber (1979) carrying out ^{14}C-labelling experiments have demonstrated that *M. rubra* was able to take up amino acids, and pilot lab experiments on a bacteria-depleted culture of *M. rubra* have provided evidence that the ciliate was able to take up leucine (B. C. C. and D. Stoecker, pers. com.). Nevertheless, in 2007, higher rates of secondary production were measured in the free-living bacterial fraction of the red water samples than in those with less or no *M. rubra* cells (data not shown; same 2007 samples for which whole water rates of secondary production are reported on Fig. 2c), suggesting that even if *M. rubra* directly affects the heterotrophic microbial production, bacteria are certainly involved, and their metabolism is likely somehow stimulated by the presence of *M. rubra* cells. Furthermore, the fact that the bacterial community structure does not vary in a manner that is consistent and dependent on *M. rubra* abundance while their rate of microbial secondary production is elevated in red waters likely reflects a metabolically flexible bacterial assemblage.

In any case, *M. rubra* blooms are clearly involved in the removal of estuarine DOC though indirect stimulation of heterotrophic bacterial activity and possibly direct heterotrophy. *M. rubra* cells are also known to excrete dissolved organic matter. Smith and Barber (1979) and Crawford et al. (1997) suggested that the peaks in bacterioplankton abundance observed after *M. rubra* blooms in the Southampton estuary were linked to the production of DOC by *M. rubra*. High *M. rubra* numbers appeared to be linked to dissolved organic components in the present study since the abundance of the ciliate was positively correlated with DOC, DON and DOP concentrations (Figs. 4a–c). Notably, DOC, like DON, was also negatively correlated with salinity (Spearman's rank correlation analysis p<0.01; rho=−0.458; n=45) whilst the ciliate abundance was not (Fig. 4a). Hence, the presence of *M. rubra* alone clearly has an important impact on DOC concentration in red waters despite the variations in DOC that may arise from its delivery from different water sources.

In summary, although the observed shift towards autotrophy associated with waters in which *M. rubra* is prevalent does not appear to influence the associated pelagic bacterial community structure, *M. rubra* blooms do offer a possible link between autotrophic and heterotrophic processes, being associated with high dissolved organic matter and showing a tendency for enhanced microbial secondary production.

Conclusion

The present study clearly showed that recurring bloom events of non-toxic mixotrophic protists, such as *M. rubra*, can have an important biogeochemical impact on their ecosystem, at least when in high abundance as in the estuarine red water patches. *M. rubra* red waters can therefore be viewed as biogeochemical hotspots of the Columbia River estuary. However, it is essential to stress that it is practically impossible to determine with accuracy the overall contribution of *M. rubra* blooms to the estuarine carbon and nitrogen budgets (i.e., on ecosystem scale rather than the red water patch scale presented here) based on sporadic water sample collection (even if in relative large numbers and at regular intervals). This is because red waters of the ciliate are ephemeral (on a daily basis) with patchy distribution. Along with this is the remaining uncertainty about the exact conditions triggering bloom formation during the day, so extrapolating to ecosystem scale data from sporadic sampling could lead to erroneous estimates. Recent advances towards profile monitoring stations that combine physical, biogeochemical, and biological sensors will be expected to play an essential role towards improving estimates of the contribution of various microorganisms to biogeochemical budgets. To this end, we started testing during the 2010–2011 *M. rubra* bloom seasons use of a Cyclop-7 phycoerythin sensor (Turner Designs, Sunnyvale, USA) attached to a profiling station in the north channel of the Columbia River estuary to estimate *M. rubra* abundance as well as other essential biogeochemical parameters. In the future, these promising high-resolution data should allow a broader temporal and spatial scale to the study of the Columbia River estuary with respect to the contribution of *M. rubra* red waters to the carbon and nitrogen budgets uncovered in the work reported herein.

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