# Red water of *Mesodinium* blooms in the Columbia River estuary contain elevated levels of *Euduboscquella* , a parasitic dinoflagellate that infects tintinnid ciliates

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## Introduction

*Mesodinium rubrum*, a mixotrophic ciliate, is known to form non-toxic red tides (red water) around the world in estuaries, fjords, and major upwelling areas along the coast (Lindholm 1985). The red color of these blooms comes from the dense accumulation of *M. rubrum* cells, which are phototactic and have several phycoerythrin-rich cryptophyte chloroplasts (Smith 1979, Fenchel 2006). The exact triggering mechanism that initiates the formation of the red water blooms is unknown. In the Columbia River estuary, *M. rubrum* blooms from late summer to early fall annually, typically in the lateral bays initially, then later in the main channels of the estuary (Herfort 2011). These blooms have been used as an indicator of environmental health and play a major role in cycling nutrients throughout the lower trophic levels of the estuary (Herfort 2012). Along with cycling nutrients through the ecosystem, the blooms also encourage rapid growth of bacterial populations and could potentially promote propagation of species that prey upon *M. rubrum,* such as the toxic dinoflagellate *Dinophysis (*Sjoqvist 2011).

Initial screening of *M. rubrum* samples for dinoflagellate species did not detect the presence of *Dinophysis*, but instead uncovered an abundance of *Euduboscquella*, a parasitic dinoflagellate known to infect tintinnid ciliates. Tintinnid ciliates play an important role in the ecosystem by preying on phytoplankton, including harmful species, and bacteria (Kamiyama 2001). *Euduboscquella*, part of the Synidiales order of dinoflagellates, is known to produce mass infections of its host during peak abundance and helps facilitate population decline. Lethal to its host, these parasitic infections help to recycle primary production in the microbial loop (Coats, 1988). Certain species of *Euduboscquella* possess in their genome a unique area of sequence polymorphism (henceforth referred to as the Unique Sequence Element, or USE) in the 28S rRNA gene (see Fig. 1), which led to the development of primers unique to Columbia River estuary *Euduboscquella* species. These PCR primers were used to study the

distribution and diversity of *Euduboscquella* in the Columbia River ecosystem and determine what role they might have in relation to *M. rubrum* blooms.

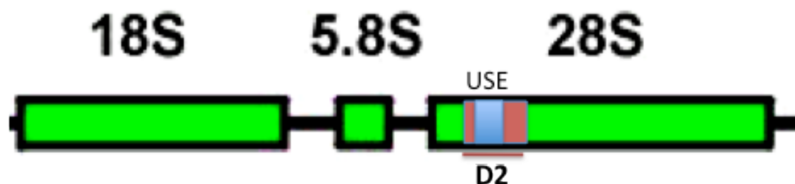


Figure 1: 28S rRNA gene map showing location of USE

## Materials & Methods

**Sampling:** Estuarine samples were collected on-shore using a 10% acid washed 2 gallon bottle that was pre-rinsed using water from the sample location. Approximately 1.5 gallons were collected and temperature and salinity were recorded at time of sampling. Samples were then filtered through two 0.22 µm sterivex filter cartridges, approximately 1.5 liters per sterivex. Samples were then preserved using RNALater, sealed, and stored in a -80° C freezer until time of extraction. Ocean samples were collected using a CTD rosette during several cruises over a period of 3 years. Samples were then processed the same way as estuarine samples and were stored in a -80° C freezer until time of extraction.

**DNA Extraction:** DNA was extracted from 0.22 um sterivex fixed with RNALater and stored in a -80° C freezer until time of extraction. Once sterivex were defrosted and cracked (using a pair of pliers), the RNALater solution was put into a 1.5 mL Eppendorf tube and centrifuged at 13,000 rpm for 2 minutes; excess RNALater was discarded. After removing the filter from the sterivex, it was cut into small pieces and placed in the 1.5 mL Eppendorf tube along with the centrifuged cells. 500 mL of Extraction buffer (10 mL 0.5 M EDTA, pH 8, 5 mL 10% SDS, and 35 mL MilliQ water) and 5 uL of Proteinase K was added. Incubated overnight in a 55° C water bath; the next day, 165 uL 5 M NaCl and 165 uL CTAB (50 mL 0.7 M NaCl and 2.5 g CTAB) was added and vortexed well. The tubes were then placed back in 55° C water bath for ten minutes, then divided into two new 1.5 mL Eppendorf tubes. 600 uL Chloroform was added and tubes were vortexed for 1.5 minutes, then centrifuged for 10 minutes. Supernatant was then transferred from both tubes into one new Eppendorf tube and extraction was then continued using the protocol from the Zymo DNA Clean and Concentrator kit (Zymo Research). Extracted DNA was then stored in a -20° C freezer until needed.

**PCR methods:** The amplification reaction mixtures contained DEPC water, 10X High Fidelity PCR Buffer (Invitrogen), 1.5 mM $MgSO_4$ (Invitrogen), 0.2 mM deoxynucleoside triphosphates (dNTPs) (Invitrogen) , 0.4 uM of each primer, and 0.1 uL of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) for a total volume of 24 uL and 1 uL of template DNA. For Dub 1 and Dub 2 PCRs (see Table 1 below), the reaction was initially denatured for 2 minutes at 92° C, then were run for 30 cycles of 94° C for 30 s, 55° C for 30 s, and 72° C for 2 min and finally was incubated at 72°C for 5 minutes. For Dub 3 PCRs (Dub2f and Dub3r) (see Table 1 below), the reaction was initially denatured for 2 minutes at 94° C, then were run for 30 cycles of 94° C for 30 s, 57° C for 30 s, and 72° C for 2 min and finally was incubated at 72°C for 5 minutes. PCR products were then run on a 1%

agarose gel (45 mL 1X TAE, 0.5 g Agarose) for 50 minutes at 90 volts and were visualized using a UV transmitter and camera.

Table 1: List of primers used

| Primer Name | Sequence | Amplifies | Expected Product Size |
|---|---|---|---|
| ORF 1 | GACTCCTTGGTCCGTGTTTCAAGA | Flanking region of the 28S USE (general) | 400 - 700 basepairs |
| ORF 2 | CGAACAAGTACCGTGAGGGAAAGATGCAAA | Flanking region of the 28S USE (general) | 400 - 700 basepairs |
| Dub C1f | GCGACGTGAACCGTCAGGGA | USE 1 in 28S region of rRNA gene | 182 basepairs |
| Dub C1r | AGGGAATCCGGCTCTCTTGGAAA | USE 1 in 28S region of rRNA gene | 182 basepairs |
| Dub 2f | GCGCCTGAAACCACTGTATTACAAGCA | Flanking region of the 28S USE *(Euduboscquella)* | 350 - 375 basepairs |
| Dub 2r | TTTCAAGACGGGTCATTTGAAACCTT | Flanking region of the 28S USE *(Euduboscquella)* | 350 - 375 basepairs |
| Dub 3r | GCACGGTTGATTGGCAGCTCCTT | USE 2 in 28S region of rRNA gene | 170 basepairs |

**Cloning & Sequencing:** PCR products were cleaned using the UltraClean PCR Clean-up Kit (MoBio Laboratories) according to manufacturer's instructions and were transformed using the TOPO TA Cloning Kit (pCR 2.1-TOPO Vector) (Invitrogen) according to manufacturer's instructions and TOP10 chemically competent cells. 75 uL of cells were plated on LB-Amp plates with 20 uL of X-gal and were grown overnight in a 37° C oven. Individual white colonies were then picked and grown again overnight in 2 mL LB liquid media and 2 uL 50mg/mL Ampicillin in a 37° C shaker. Cultures were then purified using the Manual FlastPlasmid Mini Kit (5 Prime) and DNA content was estimated using Nanodrop and diluted to 100 ng/uL (for a total volume of 6 uL) and brought over for sequencing at the OHSU Primate Center.

**Paraformaldehyde-fixed Samples and Preparation of FISH Slides:** Fluorescence in situ Hybridization (FISH) slides were prepared by filtering ~ 10 mL of a PFA prepared sample using a 0.7 uM GFF backing filter and 0.2 uM polycarbonate filter. Once filtered, filters were washed using 5 mL of 7.4 pH PBS, 2 mL of 50% ethanol (sit for 3 min), 2 mL of 80% ethanol (sit for 3 min), 2 mL of 100% ethanol (sit for 3 min), 2 mL of 0.01M HCl (sit for 10 min), and washed with 2 mL of MilliQ water (twice) and 2 mL of 100% ethanol. Hybridization buffer was prepared using the following table and probes were diluted using 1 uL probe and 11 uL of water.

Table 2: Preparation of Hybridization Buffer

| Hybridization Buffer | 2 mL total volume | 40 mL total volume |
|---|---|---|
| Formamyde 40% | 600 uL | 12 mL |
| NaCl 5M | 540 uL | 10.8 mL |
| Tris 1M | 40 uL | 800 uL |
| SDS 1% | 20 uL | 400 uL |
| Blocking reagent | 400 uL | 8 mL |
| Water | 400 uL | 8 mL |

Once prepared, the filter was cut into 4 equal sections and placed on a Parafilm-wrapped glass slide. 38 uL of Hybridization Buffer (see above table for composition) and 2 uL of probe (Dub 121, same sequence as Dub C1f primer) (Alexa555, Invitrogen) was pipetted onto each quarter and then incubated for 3 hours in a 35° C oven. While incubating, two wash buffers were prepared using the following tables:

Table 3: Preparation of Wash Buffer

| **Wash Buffer** | **10 mL total** |
|---|---|
| NaCl 5M | 224 uL |
| Tris 1M | 200 uL |
| EDTA 0.5M | 100 uL |
| SDS 1% | 10 uL |
| Water | 9.46 mL |

Table 4: Preparation of TNT Buffer

| **TNT Buffer** | **50 mL total** |
|---|---|
| Nacl 5M | 1.5 mL |
| Tris 1M | 5 mL |
| Tween 20% | 37 uL |
| Water | 43.46 mL |

Once prepared, 800 uL of Wash Buffer was placed in four tubes and filters were washed twice for 20 minutes in a 37° C water bath; once done, 800 uL of TNT buffer was put into two tubes and filters were washed for 15 minutes at room temperature in the dark. Filters were then stained with 20 uL of 0.25 ug/mL of DAPI and 10 uL of 0.033 w/v of Proflavine (5 uL of stock diluted in 45 uL of water) for 3 minutes in the dark. Filters were then washed using 1 mL of MilliQ water and mounted on a new slide with immersion oil.

**Real Time PCR (RT-PCR):** RT-PCR was used to determine the gene copy number within several samples using Dub 1 (USE number 1) primers. The reaction was done in triplicates and used a master mix of 10 uL SYBRGreen (Applied Biosystems), 8 uL of DEPC water, 0.5 uL of each primer, and 1 uL of template DNA (we do not want volumes of reagents here, we want final concentrations). The reaction was run using the same PCR protocols as described above in "PCR Methods".

## Results

**Estuarine Unique Element:** The original detection of the *Euduboscquella* unique element was found using the Open Reading Frame (ORF) primers, as described in Table 1, from a sample from Beaufort Bay in Alaska. Due to the high numbers of *Euduboscquella* sequences found during *M. rubrum* blooms from the general dinoflagellate PCR, ORF primers were used to determine if a *Euduboscquella* unique element could be found. A similar, but not homologous, USE of the same size was detected in Columbia River estuary samples during bloom periods. Consequently, new primers were designed and nicknamed "Dub 1". The results of 28S rRNA gene sequence analysis of clone libraries generated from estuarine sample DNA using "Dub 1" primers showed that *Euduboscquella* was abundant during periods of red water blooms caused by *Mesodinium rubrum*, from late August to October with peak abundance during September in Ilwaco Harbor, Bakers Bay, and Youngs Bay.

Interestingly, *Euduboscquella* only appeared in the lateral bays of the estuary, as illustrated in the map of Fig. 2. A small bloom was also detected during May and June of 2013 in Willapa Bay near Raymond, Washington; *Euduboscquella* was detected in samples from this small bloom as well. PCR results using "Dub 1" primers (USE 1) of non-bloom period estuarine samples showed no detectable *Euduboscquella* sequences; these results led to the use of FISH microscopy to visually determine if *M. rubrum* cells were being infected. Additionally, RT-PCR using the "Dub 1" primers showed that the abundance of *Euduboscquella* peaked at relatively the same time as the peak of blooms, however more analysis needs to be done.

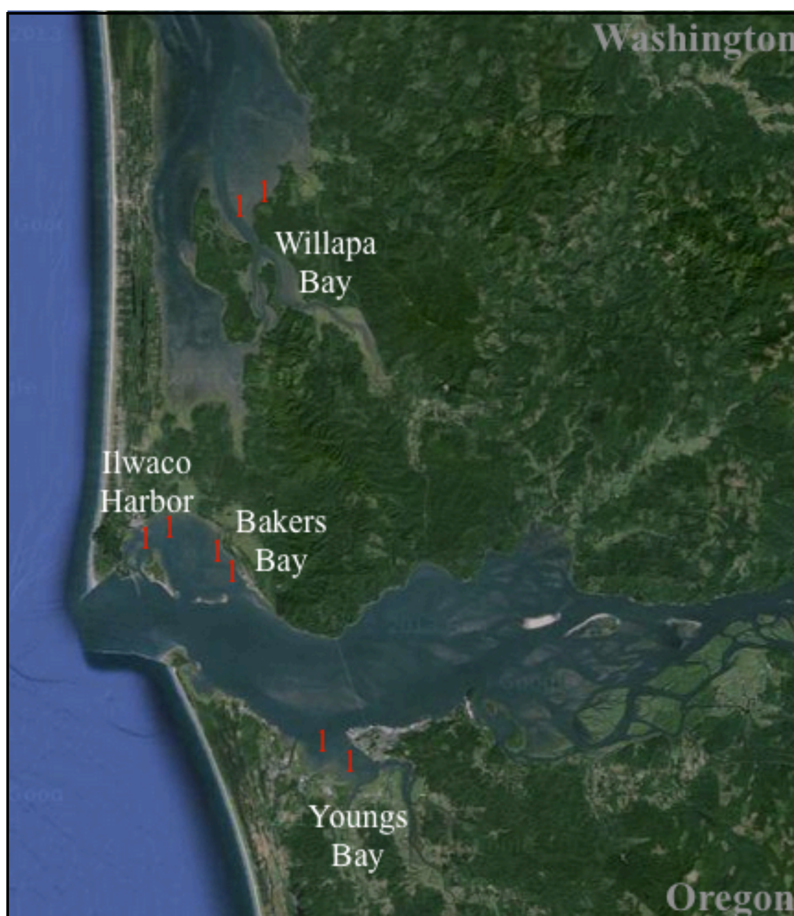


Figure 2: Map of the Columbia River estuary showing *Euduboscquella* distribution

**FISH Microscopy:** Paraformaldehyde-fixed samples from Willapa Bay, 9 May 13, and Bakers Bay, 5 September 12, were prepared using the FISH procedure as described in the "Materials & Methods" section. Three filters of an epifluorescence microscope were used to visualize *Euduboscquella* cells - Cy3, which showed the probes used, FITC to show the Proflavine staining, and

DAPI to show the DAPI staining. Both samples processed were from bloom periods of *M. rubrum*, however the DAPI staining revealed little to no Mesodinium cells infected. Instead, thousands of *Euduboscquella* were found in infected tintinnid hosts (see Fig. 3) and very few were free-living. Further identification based on DAPI staining was used to identify the loricae of tintinnid cells. Several other samples were processed as well, but the staining did not work and yielded no usable results.

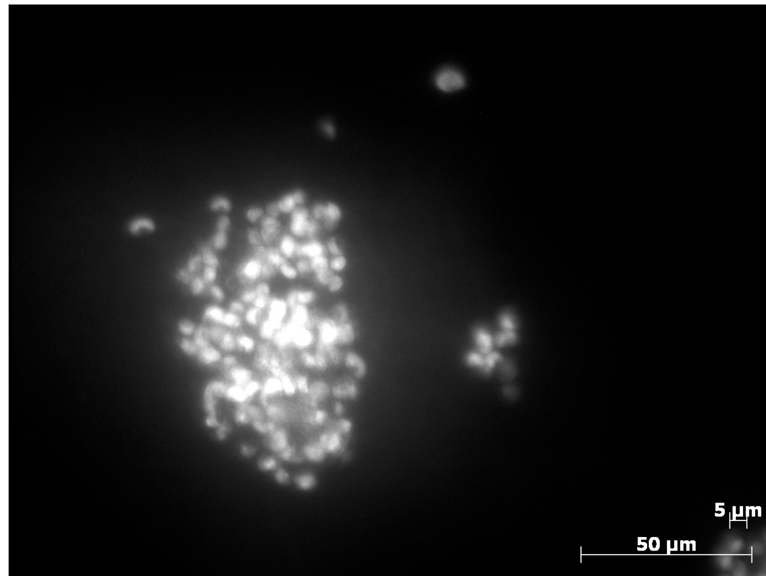


Figure 3: Epifluorescence microscope image showing infected tintinnid cell (Cy3 Filter)

**General *Euduboscquella* PCR:** New primers (Dub 2) were designed to flank the 28S region of the first unique element found (USE 1) and were initially tested on estuarine samples from pre-bloom to post-bloom months (July, August, September, and October) and 5 ocean samples from late April of 2013. PCR results and analysis of 28S rRNA clone libraries showed *Euduboscquella* was present in lateral bay estuarine samples during bloom months (late August through September) and in one ocean sample. Out of 12 sequences received, 10 aligned nearly 100% with the initial USE 1 found in previous estuarine samples, however two sequences only aligned 70% with the known USE (see Figs. 4 and 5).

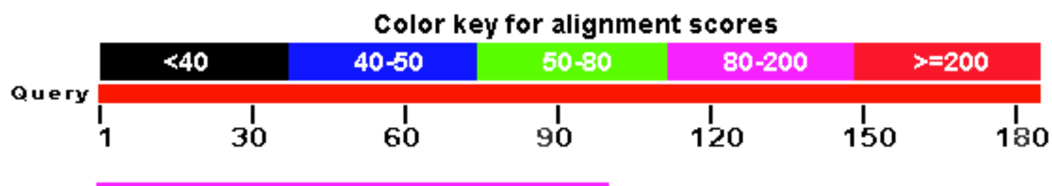


Figure 4: Alignment using NCBI BLAST showing relationship of USE 1 and USE 2

Figure 5: Phylogenetic tree showing relationship of USE 1 (Dub1) and USE 2 (Dub2_AD_)

**Oceanic Unique Element:** New primers were created from the second USE that was found in order to determine the distribution of *Euduboscquella* within the estuary. Initial PCR results of lateral bay samples during bloom periods of *M. rubrum* showed little to no detection of the second USE. However, PCR results and 28S rRNA clone libraries showed that the second element was found in oceanic samples. DNA was then extracted for 23 oceanic samples from 4 locations along the Washington and Oregon coast with varying dates (April, May, July, August, September, and November). Using the Dub 3 primers, the second USE element was found in all samples at a range of depths. Control PCR was also run on these samples using the “Dub 1” primers (USE 1) and “Dub 2” primers (flanking the unique element). Detection of the Columbia River estuary *Euduboscquella* was found in all 23 samples (see Fig. 6) and was confirmed to be *Euduboscquella* using the “Dub 2” (flanking) primers.

Figure 5: Map of ocean samples showing detection of USE 1 (indicated by 1) and 2 (indicated by 3)

## Conclusions

Initial PCR results and analysis of 28S rRNA clone libraries of estuarine samples using primers specific to the first Unique Sequence Element showed a possible relationship between *M. rubrum* and *Euduboscquella*. Results showed that *Euduboscquella* was only detected in bloom month samples (late August to September), with the highest detection found during the peak of blooms. FISH microscopy, however, neither confirmed nor refuted the theory that *Euduboscquella* was infecting *M. rubrum* - of the two samples processed, both showed thousands of *Euduboscquella* cells inside infected tintinnids, but none infecting *M. rubrum*. It is possible that *Euduboscquella* is not infecting *M. rubrum* as previously thought, but is found in conjunction with blooms due to an increase in tintinnid populations. *M. rubrum* blooms can lead to an increase in bacteria and predator populations and, as previously mentioned, tintinnids are known to prey on bacteria. The increase in tintinnid populations likely increases *Euduboscquella* populations as they are a known parasite. Thus, *Euduboscquella* is likely a factor in the eventual decline of tintinnid populations and is likely why there was little to no detection of *Euduboscquella* after the end of *M. rubrum* blooms.

Primers that flanked the unique variable region of the 28S rRNA gene were created to confirm that the USE being amplified was *Euduboscquella*. Initial analysis of the clone libraries showed that, out of 12 samples, 10 aligned nearly 100% with the first USE found. However, analysis of 2 ocean samples showed a deviation of ~ 30% with the first USE. New primers were created specific to this new element and PCR results showed that it was only found in oceanic samples, not in the estuary like USE 1, and is present year-round. Control PCR tests using the USE 1 primers, Dub 1, were run and detection was shown in every sample. This helps support the theory that *Euduboscquella* is not infecting *M. rubrum*, but was present in high numbers due to an increase of tintinnids.

Future work will hopefully include more FISH microscopy of non-bloom estuary samples to identify both tintinnids and *Euduboscquella*, more sequence analysis of 28S rRNA clone libraries, and more sampling of both the ocean and estuary.

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