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Analysis of *in situ* manganese(II) oxidation in the Columbia River and offshore plume: Linking microbial community structure to active biogeochemical cycles

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Abstract

The Columbia River is a major source of dissolved nutrients and trace metals for the west coast of North America. A large proportion of these nutrients are sourced from the Columbia River Estuary where coastal and terrestrial waters mix and resuspend particulate matter within the water column. As estuarine water is discharged off the coast it transports the particulate matter, dissolved nutrients and microorganisms forming nutrient rich and metabolically dynamic plumes. In this study, bacterial manganese oxidation within the plume and estuary was investigated during spring and neap tides.

The microbial community proteome was fractionated and assayed for Mn oxidation activity. Proteins from the outer membrane and the loosely bound outer membrane fractions were separated using size exclusion chromatography and Mn(II)-oxidizing eluates were analyzed with tandem mass spectrometry to identify potential Mn oxidase protein targets. Multi-copper oxidase (MCO) and heme-peroxidase enzymes were identified in active fractions. T-RFLP cluster analysis indicates that the organisms oxidizing the most Mn(II) were sourced from the Columbia River estuary and nearshore coastal ocean. These organisms are producing up to 10 fM MnO₂ cell⁻¹ day⁻¹. Evidence for the presence of Mn(II)-oxidizing bacterial isolates from the genera *Aurantimonas*, *Rhodobacter*, *Bacillus*, and *Shewanella* was found in T-RFLP profiles. Q-PCR was used to quantify the gene copies of the heme-peroxidase, *Aurantimonas* SSU rRNA and total bacterial SSU rRNA gene copies. The probes used suggested that *Aurantimonas* could only account for 1.7% of heme-peroxidase genes quantified suggesting that peroxidase driven manganese oxidation capabilities are widespread throughout other organisms in this environment.

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Keywords

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Introduction

The Columbia River is responsible for the largest input of fresh water to the California Current system and is a major source of dissolved trace metals and nutrients (Barnes *et al.*, 1972). The Columbia River estuary is characterized by strong tidal cycles, high turbidity and dynamic mixing where the coastal waters invade the estuary. Sediments are dispersed into the water column during tidal events forming estuarine turbidity maximum (ETM) events (Gelfenbaum, 1983; Nichols and Biggs 1985; Jay and Musiak 1994; Berner and Berner 1996). ETM events occur at locations where the residual flow is zero (tidal inflow equals river outflow) and can resuspend large volumes of nutrient-rich sediment into the estuarine water column (Baross *et al.*, 1994; Prah1 *et al.* 1997; Crump *et al.*, 1998). During neap tides, any sediment is generally sourced from upriver and there is a low sediment distribution in the water column, but during spring tides estuarine sediments are strongly dispersed into the water column over large distances (Small and Prah1, 2004).

With the outgoing tide, the estuarine waters mix with the coastal waters and form a lower salinity plume that carries the dissolved metals, nutrients and sediments from the estuary out into coastal waters (Hill and Wheeler, 2002; Lohan and Bruland, 2006; Aguilar-Islas and Bruland, 2006)). The plume itself is vertically stratified with less dense lower salinity waters at the surface and higher density saline waters at depth. The zone of mixing between coastal and estuarine waters occurs at the surface (up to approximately 10m depth at most) (Hickey, 1998). In general, the plume moves south in the summer (upwelling conditions) and is more diffuse, whereas in the winter (downwelling conditions) the winds tend to push the plume north and closer to the coast (Barnes *et al.*, 1972; Landry *et al.*, 1989; Hickey *et al.*, 2005).

The plumes not only contain particulate matter and dissolved nutrients but they also contain microorganisms (Small and Prah1, 2004). These microorganisms can be sourced from upriver (terrestrial and aquatic), estuarine or coastal pools (Crump *et al.*, 1999). Previous studies have demonstrated that during ETM events there is an increase in bacterial activity (Baross *et al.*, 1994; Crump and Baross 1996; Crump *et al.*, 1998). Bacterial RNA concentrations (used as a proxy for the abundance of live organisms) also indicate an abundance of live and potentially metabolically active microorganisms in the plume (e.g., for samples from the May-June 2008 R/V Wecoma cruise; M. Smit, 2009, pers. comm.).

The Columbia River Estuary is a major source of dissolved and particulate manganese that is carried offshore by the plume (Bruland *et al.*, 2008). Manganese has three environmentally relevant oxidation states, II, III and IV. Soluble Mn(II) and insoluble Mn(IV) oxides are commonly recognized as the main forms in aquatic environments but Mn(III) is now recognised as being very important (Trouwborst *et al.*, 2006). Mn(III) is highly reactive and thermodynamically unstable unless chelated or complexed with ligands such as pyoverdine and pyrophosphate, or structurally incorporated into Mn(III/IV) oxides (Kostka *et al.*, 1995;

Parker *et al.*, 2007; Tebo *et al.* 2004). Mn(III) complexes may be an important constituent of the dissolved and particulate Mn found in the Columbia River Estuary along with Mn(IV) oxides which are common constituents of the estuarine sediments (Klinkhammer and McManus, 2001).

Manganese plays an essential role in many geochemical and biological processes. Manganese oxides have a strong affinity and sorptive capacity for many trace elements (and heavy metals such as Co, Pb, Cu, Cd and Ni) (Huang, 1991; Tebo *et al.*, 2004) and can therefore heavily influence the solubility and biological availability of trace metals and pollutants in the Columbia River. Manganese oxides also serve as an electron acceptor in anaerobic sediments coupled with the oxidation of organic matter. Dissolved Mn(II) from biological Mn(IV) reduction or water-rock interactions is biologically available to planktonic microorganisms. Manganese is an essential biological micronutrient serving as the catalytic center of photosystem II and oxidative stress enzymes. Manganese(II) can also be cycled to Mn(IV) by various Mn(II)-oxidizing bacteria and fungi (Tebo *et al.*, 2005).

In the pH range of oxic natural waters (pH 6–8.5), the chemical oxidation of Mn(II) is generally slow. The Mn cycle is accelerated enzymatically by microorganisms who can increase the Mn(II) oxidation rates by up to 4–5 orders of magnitude (Nealson *et al.*, 1988; Tebo, 1991; Wehrli *et al.*, 1995). Cultured bacteria that oxidize Mn(II) are phylogenetically diverse and include alpha-, beta- and gamma-*Proteobacteria* such as *Leptothrix discophora* and *Pseudomonas putida* GB-1, the low GC Gram-positive bacteria (*Firmicutes*) such as *Bacillus* sp. strain SG-1, and the *Actinobacteria*. Manganese oxidizers within the alpha-*Proteobacteria* include *Roseobacter* spp., *Pedomicrobium* sp. ACM 3067, *Erythrobacter* sp. strain SD-21, *Aurantimonas manganooxydans* strain SI85-9A1, *Sulfitobacter* spp., *Methylophilus* spp., and *Rhodobacter* spp. (Caspi *et al.*, 1996; Larsen *et al.*, 1999; Francis *et al.*, 2001; Tebo *et al.*, 2005; Templeton *et al.*, 2005; Hansel and Francis 2006; Anderson *et al.*, 2009a). Organisms isolated from the Columbia River estuary and off the Oregon coast include species from the genera *Aurantimonas* and *Rhodobacter*, *Bacillus*, and *Shewanella* (Anderson *et al.*, 2009a; Bräuer *et al.*, 2010).

From a molecular perspective, only multi-copper oxidases (MCO's) from *Bacillus* spp. and heme containing Mn(II)-oxidizing peroxidases (Mop) from *Aurantimonas* and *Erythrobacter* species have been directly linked to active Mn-oxidation in bacteria (Dick *et al.*, 2008, Anderson *et al.*, 2009b). Other MCO's involved in Mn(II) oxidation have been genetically identified in *Pseudomonas putida* strains MnB1 and GB1 (*cumA*), *Leptothrix discophora* SS-1 (*mofA*), and the alpha-*Proteobacterium* *Pedomicrobium* sp. ACM 3067 (*moxA*) (Ridge *et al.*, 2007). Mn(II)-oxidizing heme peroxidases are best known to occur in fungi and are a relatively new group of proteins to be linked to bacterial Mn(II) oxidation (Anderson *et al.*, 2009b). A similar Mn(II) oxidation mechanism to the fungal peroxidases has been postulated for a catalase/peroxidase protein from *Mycobacterium* (Magliozzo and Marcinkiewicz, 1997) and catalase coupled to peroxide was suggested to be involved in Fe and Mn oxidation in *Arthrobacter* (Dubinina, 1978).

Despite the well-recognized environmental importance of manganese, major questions concerning the identity, physiology, and ecology of the key microorganisms that drive the

oxidative segment of the Mn cycle remain unanswered. This study investigates the *in situ* bacteria that are actively involved with manganese oxidation. Microorganisms from water samples taken within the Columbia River estuary and plume were collected and their proteins were fractionated to collect subsamples with active Mn-oxidizing proteins. These proteins were identified by tandem mass spectrometry. Genomic DNA was extracted from the same Mn(II)-oxidizing bacterial community and was analyzed using T-RFLP and Q-PCR. The overall goals of this project were to identify patterns in the microbial community structure, find evidence for active *in situ* bacterial Mn(II) oxidation, and to identify proteins and genes known to be involved with Mn(II) oxidation to determine a direct relationship between the bacterial community and the process of *in situ* bacterial Mn(II) oxidation.

Materials and methods

All aqueous solutions were prepared using ultrapure water from a MilliQ water system (18 M Ω -cm resistivity) and all chemicals used were ACS reagent grade, unless otherwise stated.

Shipboard

Sample Collection—A total of 10 water samples were collected on the May-June 2008 R/V *Wecoma* cruise from the Oregon and Washington coasts, the Columbia River, river plume and estuary (Fig. 1). Samples were retrieved using a SeaBird CTD system equipped with a transmissometer, fluorometer, PAR, O₂ probe, altimeter and a SBE Carousel sampler with twelve 10 L Niskin sampling bottles. Each sample was labelled with a CTD cast number and sampling depth.

Five of the samples collected (10 L sample volumes) were designated for genomic DNA extraction only, these being: E2_11m, C1_15m, C2_51m, C3_2m, and O1_22m (Fig. 1). The other five samples collected (60 L sample volumes) were designated for both genomic DNA and protein extraction. P1_2m and P2_19m were collected on June 1st 2008 4 days after the neap tide cycle in the plume region while P3_2m and P4_102m were collected on June 3rd 2008 during the spring tidal cycle in the plume region. E1_12m was collected on June 2nd 2008 within the estuary (~20km from the river mouth) in front of the incoming tidal salt wedge. Fifty milliliter aliquots of water were reserved from each of these samples for culturing and most probable number (MPN) assays.

To collect biomass for genomic DNA extraction the samples were filtered through Sterivex filter cartridges (Millipore, Billerica, MA). Duplicate Sterivex filters were used for each sample and water was simultaneously passed through the filters until they clogged. The volume filtered varied with only 0.5 to 0.92 L of water filtered for the sediment rich estuarine samples and between 2.9 to 15 L filtered for coastal samples. The Sterivex filters were immediately frozen at -80 °C in sterile 50 ml centrifuge tubes until DNA extraction onshore. In order to get enough biomass for protein extractions, 40 L samples of water were concentrated to 300 to 400mL volumes using a tangential flow filtration (TFF) system with a 0.22 μ m filter. Fifty millilitre aliquots of the concentrated samples were reserved for shipboard whole cell Mn(II) oxidation assays with the remainder frozen at -80 °C in sterile 50 ml centrifuge tubes for protein analysis onshore.

Culturing and MPN—Aboard the ship, culturing plates were inoculated along with a most probable number (MPN) assay to determine the number of heterotrophic bacterioplankton and the proportion of those that could oxidize manganese. The non-concentrated water samples underwent two different 10-fold serial dilutions (10^{-1} to 10^{-8}) in K Medium (Tebo *et al.*, 2006) where one dilution series contained 100 μ M MnCl_2 and the other did not. From each dilution 133 μ L was aliquoted into each of 6 wells in duplicate 96-well plates where one half of the plate was the series with manganese and the other half was the series without manganese. The plates were incubated at room temperature for a period of 2 weeks for one of the duplicate plates and 4 weeks for the other. Onshore, after incubation, 100 μ L of a 0.04% solution of a luekoberbellin blue solution (LBB) (Tebo *et al.*, 2006) was added to the wells containing MnCl_2 to detect the formation of manganese oxides while 50 μ L of a 0.3% solution of Iodonitotetrazolium salt (INT) was added to the other wells to assay cell growth. After overnight incubation the plates were visually quantified with positive wells being either red for INT or blue for LBB. The number of positive wells for each dilution was entered into a downloadable MPN calculator (Version VB6) (www.i2workout.com/mcuriale/mpn/index.html). For culturing, 20 μ L of the undiluted, the 10^{-1} , 10^{-2} , and the 10^{-3} dilutions were spread onto agar plates of K medium, M medium, Lept. medium and 25% M medium. K and M-media are seawater based while 25 % M and Lept. medium were used for culturing organisms from brackish or fresher water environments (Tebo *et al.*, 2006).

Whole Cell Assay—A whole cell Mn(II) oxidation assay was performed at sea for the 5 samples collected for protein analysis. Bacterial cells from 22.8 mL of the TFF concentrated samples were pelleted in a microcentrifuge. The pellet was resuspended in 4ml of 10 mM HEPES buffer (pH 7.6) with 50 mM NaCl, 100 μ M MnCl_2 , and 5 mM Na-Pyrophosphate (PP_i) and then split into two 2 mL fractions. One of the fractions was killed with a final concentration of 0.02% Na-azide to act as a control. The live fractions were measured every 15 minutes for 24 hours by scanning with an Ocean Optics (Dunedin, FL) spectrophotometer and integrating the area between 245 and 270 nm to monitor the formation of a Mn(III)-pyrophosphate complex which adsorbs at 258 nm. The azide-killed controls were measured at both the start and end of the experiment. After 48 hours 250 μ L of LBB solution was added to all tubes to quench the reaction followed by an overnight incubation to allow the LBB to react with the Mn(III)- PP_i complexes. The LBB absorbance at 620 nm was recorded at sea. Onshore, the samples were aliquoted into 96-well plates and the LBB absorbance was re-measured at 620 nm using a plate reader (Molecular Devices, Sunnyvale, CA).

Laboratory analysis

Cell Fractionation—Cells from the mixed microbial community were fractionated using a combination of protocols from Myers and Myers (1992), Gaspard *et al.* (1998) and DiChristina *et al.* (2002). Five different protein fractions were recovered, these being: secreted (proteins in the seawater at *in situ* concentration), loosely bound outer membrane (LBOM), soluble (periplasmic and cytoplasmic), inner membrane (IM) and outer membrane (OM).

The concentrated biomass samples were thawed overnight at 4 °C and then particulate material (sediments and biomass) was pelleted at 4 °C at $10,000 \times g$ for 20 minutes. The supernatant (secreted proteins) was decanted into a separate container, pre-filtered through sterile Whatman paper, followed by filtration through a 0.22 μm Millipore Stericup and then ultra-filtered to a volume of ~5 mL using a 400 mL Amicon (Millipore) stirred filtration cell with a 47 mm diameter 10 KD NMWL cellulose filter (Millipore).

The pellet was resuspended in 20 mL of 100 mM HEPES buffer with 100 μM ascorbate, added to reduce any Mn oxides to Mn(II). Cells were pelleted by centrifugation and the supernatant was collected. The pellet was resuspended, stirred vigorously in 20 mL of a high salt Tris buffer (100 mM Tris, pH 7.5, 1 M KCl, 1 mM DTE) for 4 hours at 4 °C and then centrifuged again. The high salt buffer supernatant was collected and added to the supernatant from the ascorbate wash. These combined supernatants were pre-filtered through sterile Whatman paper, followed by filtration through a 0.22 μm Millipore Stericup and then ultra-filtered to a volume of ~5 mL using the Amicon stirred filtration cell with a 10 KD NMWL filter. The resulting concentrated solution was considered the loosely bound outer membrane (LBOM) protein fraction. The pellet was then resuspended and washed in 6 mL of 10mM Tris HCl (pH 8.1) buffer followed by 3 rounds of lysis using a French Press. The membrane and soluble fractions were then separated by ultracentrifugation at $200,000 \times g$ for 1 hour at 4 °C using a Beckman TLA 100.1 rotor (Beckman Coulter, Fullerton, CA). The supernatant was collected and was considered the soluble (periplasmic and cytoplasmic) protein fraction.

The pelleted outer and inner membrane fractions were separated using Triton X-100 to solubilize the inner membrane proteins. The pellet was first resuspended in 3 mL of 10 mM HEPES buffer (pH 7.5), with 10 mM MgCl_2 and then equilibrated overnight at 4 °C. The resuspended pellets were then equilibrated to 22 °C, and Triton X-100 was added to a final concentration of 2.0 % (vol/vol) followed by a 10 minute incubation at room temperature. The tubes were put on ice, and then ultra-centrifuged at $100,000 \times g$ for 2 hours at 4 °C. The supernatant was collected and was considered the inner membrane protein fraction. The resulting pellet, containing the Triton X-100 insoluble material, was resuspended in 3 mL of 10mM Tris-HCl solution (pH 8.1). A 1/10 volume equivalent of lysozyme (final concentration 6.4 mg mL^{-1}) was added to the solution and allowed to incubate for 20 minutes. The Triton X-100 insoluble material was pelleted again by ultracentrifugation. The resulting pellet was resuspended in 10 mM HEPES buffer (pH 7.5) and was considered the outer membrane protein fraction.

Once all of the fractions were collected they were dialyzed overnight in 10mM HEPES buffer (pH 7.5) using 6000–8000 MWCO dialysis tubing. After dialysis, each fraction was then concentrated further to a final volume of 1.6 mL using Millipore Biomax Ultra centrifugal filters with a 10 KD NMWL cutoff. From this 1.6mL, 100 μL was used for fraction activity assays and the remainder was used for FPLC protein purification.

Fraction Activity Assays—An overnight activity assay was performed to determine if any of the sub-cellular protein fractions from the *in situ* microbial community were actively oxidizing manganese. A 100 μL volume of each fraction was added to 890 μL of HEPES

buffer (100 mM , pH 7.5) with 50 mM NaCl and 100 μ M of MnCl₂. Control tubes were also prepared that did not contain MnCl₂. Six 150 μ L aliquots were then added to separate wells in 96-well plates and the plates were incubated at room temperature overnight. After incubation, 150 μ L of LBB was added to each well and allowed to react for 1 hour with any Mn-oxides produced. The difference in absorbance at 620 nm between the controls and samples was recorded and any proteins from fractions that had activity were then separated by size exclusion using FPLC.

Fast Protein Liquid Chromatography (FPLC)—An ÄKTA FPLC system (G.E. Healthcare, Little Chalfont. UK) with a HiPrep 16/60 Sephacryl S-200 high-resolution column (G.E. Healthcare) was used to separate the proteins by size from the active sub-cellular fractions. The FPLC consisted of a UPC-900 monitor, P-920 Pump, M-925 Mixer and INV-907 valve and a FRAC-950 Fraction collector. The column was initially cleaned using 2 column volumes (CV) of H₂O, followed by short-term storage in 20 % ethanol, washing again with 2 CV of H₂O and then 5 CV of 20 mM HEPES buffer (pH 7.8) with 150 mM NaCl.

For each sample, 2 CV of buffer (20mM HEPES buffer, pH 7.8, with 150 mM NaCl) were run at 0.5 mL per minute through the column to clean and equilibrate the column prior to sample injection. A 2 mL injection loop was used with a 1.5 mL sample volume. The sample was eluted through the column with 1.5 CV of buffer and 1mL fractions were collected in 2 mL volume deep well 96-well plates. A 90 μ L aliquot of each eluted fraction was added to a fresh 96-well plate and 10 μ L of 1 mM MnCl₂ was added to identify eluted fractions that had active Mn oxidation post FPLC. Those eluted fractions that turned blue after the addition of 100 μ L of LBB were then pooled together and the fractions were desalted and concentrated to a final volume of 50 μ L using Millipore Biomax Ultra 10 KD NMWL spin filters.

Column contamination was tested after all samples were run by washing as described above and injecting a blank sample followed by a sample containing LBOM proteins extracted from a 1 L culture of the Mn-oxidizing alpha-Proteobacterium *Aurantimonas manganoxydans* strain SI85-9A1. Two further blank samples were injected after the *Aurantimonas* proteins. For each contamination test sample all eluted fractions were collected, pooled and concentrated to a final volume of 50 μ L using 10 KD NMWL centrifugal filters and tested for Mn(II) oxidation with LBB.

Tandem Mass Spectrometry—Tandem Mass Spectrometry (MS/MS) was used to identify the proteins from the active fractions pooled from the FPLC and any proteins recovered in the contamination test samples. The protein concentration in each sample was estimated using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) and was then diluted and dried in a Speedvac (Thermo Scientific) to a final dry mass of ~20 μ g. These dried samples were resuspended and digested using trypsin at the Shared Protein Resource facility at Oregon Health & Science University (OHSU). The samples were rehydrated in a digestion buffer and 2 μ L of dithiothreitol (DTT) solution was added and were incubated for 15 minutes at 50 °C followed by the addition of 1 μ L of isoamyl alcohol solution. The samples were then incubated in the dark at room temperature for 15

minutes and then an additional 4 μL of DTT solution was added followed by incubation at room temperature for another 15 minutes. Enough trypsin was then added to ensure a 1:25 enzyme to substrate ratio in solution along with water to achieve a final volume of 40 μL . The samples were then centrifuged and left to incubate overnight at 37° C. After incubation, formic acid (2 μL of 88 % acid) was added to halt the digestion process and the samples were frozen until analysis.

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot). Mascot was set up to search NCBI assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 1.2 – 1.4 Da, U-1 of selenocysteine, b+1 of asparagine/aspartic acid, z+1 of glutamine/ glutamic acid and iodoacetamide derivative of cysteine were specified in Mascot as fixed modifications. Oxidation of methionine was specified in Mascot as a variable modification.

Scaffold (version Scaffold_2_01_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 50.0% probability as specified by the Peptide Prophet algorithm (Keller *et al.*, 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. For the purpose of grouping proteins by potential function, a probability of >90% with at least 2 identified peptides was used. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al.*, 2003). Proteins that contained similar peptides but could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Protein fragments were queried against the environmental protein database and the draft genome database of *Aurantimonas* sp. strain 9A1.

DNA Extraction—Total genomic DNA from each duplicate sample was extracted using the FastDNA Spin Kit for soil (MP Biomedical Sciences, Solon, OH). Each Sterivex filter was broken open with sterilized pliers and the filter was removed and cut in half using sterile forceps and scalpels. The filter with all attached biomass was distributed between two bead beating extraction tubes and genomic DNA was extracted following the manufacturer's protocol. Purified genomic DNA from the duplicate extractions was pooled together, cleaned, desalted and concentrated using 100 KD Montage PCR centrifugal filter devices (Millipore). The concentration of DNA was measured using a Nanodrop ND-1000 spectrophotometer and each sample was then diluted to obtain a final concentration of ~10 ng μL^{-1} with filter sterilized 10 mM Tris, pH 8.0. DNA was extracted from pure Mn(II)-oxidizing bacterial isolates (subcultured from the CFU plates on the same medium that they were isolated on) using the same DNA extraction kit but with a cell pellet from a liquid culture as the starting material.

DNA Amplification and T-RFLP Preparation—Samples of extracted DNA were prepared for analysis according to the TRFLP protocol outlined in (Davis and Moyer, 2008). Triplicate PCRs were performed using Bacterial domain specific primers and 50 ng of total DNA. The specific primers used for bacteria were 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (YGRTACCTTGTTACGACTT). The forward primer was labeled with the fluorochrome 6-FAM (6-carboxyfluorescein) on the 5' end. The resulting PCR products

were visually assayed for size using a 1% agarose gel with a 1-kb ladder DNA size standard. The fluorescently labeled PCR products from the triplicate reactions were pooled together, desalted using a 100 KD Montage PCR centrifugal filter device (Millipore) and eluted in 150 µl of molecular biology grade water (MoBio, Carlsbad, CA).

The DNA extractions were digested with the enzymes *Hae* III, *Hha* I, *Alu* I, *Msp* I, *Rsa* I, *Hinf* I, and *Bsu* I (New England Biolabs). The resulting restriction fragments were desalted using a Sephadex G-75 column and dehydrated. The restriction fragments were resuspended in 15 µl of Hi-Di deionized formamide with Genescan ROX-500 internal size standard (Applied Biosystems, Foster City, CA). The digests were then denatured by heating at 95 °C for 2 minutes and were separated with capillary electrophoresis on an ABI 3100 Genetic Analyzer fitted with a 50 cm capillary filled with POP6 polymer (Applied Biosystems). Each T-RFLP digestion was separated and visualized at least twice to ensure reproducibility and reliable results. T-RFLP fingerprints were analyzed using the software Bionumerics from Applied Maths.

The SSU rRNA gene from pure cultures was amplified using the 27F and 1492R primers as defined above but without the 6-FAM label. The PCR products were cloned into Invitrogen pCR 4-TOPO cloning vectors. Plasmid DNA from 10 transformants was extracted, the SSU rRNA gene inserts were checked on 1% agarose gels and the inserts from 2 plasmids were sequenced using M13 primers.

Q-PCR—Two separate Q-PCR reactions were performed with the DyNAmo™ Flash SYBR® Green qPCR Kit (New England Biolabs, Beverly, MA), the first with *Aurantimonas* specific SSU rRNA primers, the second with degenerate primers designed for Ca-binding heme peroxidases. Alignment of thirteen 1400 bp *Aurantimonas* SSU rRNA gene sequences was performed using the ARB software environment (Ludwig *et al.*, 2004). Two unique *Aurantimonas* probes were designed with ARB using the Silva release 95 SSU ref database (Pruesse *et al.* 2007) these being: 148–155F 5'-GATAGCTCCGGGAAACT-3' and 301–282R 5'-CTCTTAGACCAGCTAWGGAT-3' (where W will substitute for A or T), giving a Q-PCR fragment of 138 bp. To get the proportion of *Aurantimonas* SSU rRNA gene copies among the total bacteria SSU rRNA gene copies the universal primers, 357F 5'-CTCCTACGGGAGGCAGCAG-3' and 536R 5'-GTATTACCGCGGCTGCTGG-3' were used giving a Q-PCR fragment of 179 bp (Fierer, 2005). For the Ca-binding heme peroxidase, 17 orthologous DNA sequences from organisms representing 10 different genera (*Arthrobacter* sp. FB24, *Aurantimonas manganooxydans* SI85-9A1, *Erythrobacter* sp. SD-21, *Fulvimarina pelagi* HTCC2506, *Methylobacterium chloromethanicum* CM4, *Methylobacterium chloromethanicum* CM4 ctg66, *Methylobacterium chloromethanicum* CM4 ctg72, *Methylobacterium extorquens* PA1, *Mesorhizobium* sp. BNC1, *Rhodopseudomonas palustris* BisA53, *Rhodopseudomonas palustris* BisB5, *Roseobacter* sp. AzwK-3b (two copies), *Roseobacter* sp. MED193, *Roseovarius* sp. TM1035, *Pseudomonas putida* F1, *Pseudomonas putida* GB-1 and *Pseudomonas putida* KT2440) were manually aligned using the multiple alignment program Bioedit (Hall 1999). The alignment was used to create an ARB database that was then used to design universal primer sets. The forward primer was 1354–1372F 5'-CACCA YRTSTTCCAYWSCG-3' (where Y is an equal mixture of C and T, R is an equal mixture of A and G, S is an equal mixture of G and C and W is an

equal mixture of A and T), and the reverse primer was 1584-1564R 5'-GCTGRTACTGCATYTCSGT-3' giving a Q-PCR fragment of ~210 bp covering an area within the heme binding region of the gene.

Each set of primers was optimized for temperature and run conditions on an Applied Biosystems StepOnePlus Real-time PCR system over a range of annealing temperatures from 56 to 66 °C. Melt curves were used to determine the optimum temperature for fluorescence data acquisition where Q-PCR fragments would be intact but interfering signal would be eliminated. Standards were prepared using plasmids containing the SSU rRNA gene from *Aurantimonas manganoxydans* sp. SI85-9A1 (Invitrogen pCR 4-TOPO cloning vector) and the Ca-binding heme peroxidase (ZP_01225898) from *Aurantimonas manganoxydans* sp. SI85-9A1 (Fermentas pJET 1.2 cloning vector). Each reaction contained: 10 µl of 2X DyNAmo Flash master mix, 7.4 µl Water, 0.2 µl Forward primer (50 µM stock), 0.2 µl Reverse primer (50 µM stock), 0.2 µl 50X ROX, 2 µl of DNA. The run conditions for the *Aurantimonas* and universal primers were 95 °C for 7 minutes followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. For the peroxidase primers the annealing and elongation steps were combined and performed at 58 °C for 30 seconds followed by data acquisition at 82 °C for 10 seconds.

Results and Discussion

Microbial community characteristics

The microbial physiology and community diversity of the Columbia River, estuary and coastal waters is strongly influenced by the dynamic chemical and physical properties of the environment (Crump *et al.*, 1999). Within the Columbia River estuary and plume, tidal flux and variations in associated salinity (practical salinity units, PSU) are correlated to the basic physiology of culturable organisms and community genetic fingerprints (Fig. 2). Approximate colony-forming unit (CFU) counts of bacteria growing on culture plates with different salinities demonstrated that growth media with a salinity that most closely matched the environment where the samples were collected supported the highest numbers of heterotrophic bacteria (data not shown).

The most probable number (MPN) assay reflected heterotrophic growth only and suggested culturable cell numbers of 2.4×10^3 to 2.5×10^4 cells mL⁻¹ for fresh water environments up to 3.1×10^4 cells mL⁻¹ for surface coastal waters and 1.2×10^3 cells mL⁻¹ for deep (>19 m) coastal waters (Table 1). If the MPN data is compared to the Q-PCR data produced with universal bacterial primers (assuming an average of three SSU rRNA gene copies per cell), between 1 and 10 % of the bacterial community was culturable except for sample CTD156_2m where 31 % of the organisms are potentially culturable (Table 1). The Q-PCR estimates for the total bacterial numbers for the estuary are on the same order of magnitude as the numbers of cells counted by Crump *et al.*, (1999).

Of the culturable heterotrophs, a maximum of only 0.029 % (7.25 cells mL⁻¹) could oxidize manganese representing a mere 0.00024 % of the total bacterial community based on Q-PCR data. The variation in MPN numbers between the samples considered freshwater, P1_2m (2 PSU) and E1_12m (0.3 PSU), is probably related to a higher proportion of particle

attached bacteria being included for sample E1_12m as this sample had the highest sediment load of all samples. Particle attached bacteria are thought to contribute up to 90% of the heterotrophic bacterial activity (Crump and Baross, 1996; Crump *et al.*, 1999).

DNA extracted from all the samples was analyzed using T-RFLP community fingerprint profiling. Clustering of genetic fingerprints from the microbial communities also follows salinity (PSU) with assemblages from the estuary and plume grouping together, deep-water assemblages grouping together and coastal ocean sequences grouping together (Fig. 2). These clusters indicate the nearshore coastal ocean and associated currents are heavily influenced by nutrients and materials (biomass and particulate matter) discharged by the Columbia River. These clusters also represent a transfer of microbial biogeochemical cycling capabilities from the dynamic estuarine ecosystem to the coastal ocean. This idea is supported by previous chemical studies of the Columbia estuary and plume where transfer of, chlorophyll, dissolved and particulate organic matter and nutrients such as iron, manganese, silicic acid and nitrate in the coastal ocean have been directly traced to the estuary (Klinkhammer *et al.*, 2000; Small and Prahl, 2004; Bruland *et al.*, 2008). It is unknown how long these microbial driven cycles continue off the coast considering the mortality of the organisms involved is closely associated with changes in water chemistry (Troussellier *et al.*, 2002), but the nutrients transported offshore will be affected by the microorganisms transported with them. For manganese, this means Mn(II) will continue to be biologically oxidized to the more geochemically reactive oxidation states of Mn(III) and Mn(IV).

The results from a study by Aguilar-Islas and Bruland (2006) suggest that the process responsible for a 4-fold variability in the dissolved Mn concentrations in the Columbia River plume must take place within the estuary or just outside the river mouth. They also suggest that elevated dissolved manganese concentrations are sourced from the estuary during spring tides and with increased suspended sediment content. If variation in dissolved Mn is attributed to Mn-reducing bacteria (as suggested by Klinkhammer and McManus, 2001) and if we assume that the majority of the bacteria in the estuary system are particle associated (Crump and Baross, 1996) then spring tidal influx into the Columbia River estuary will resuspend large amounts of sediment containing high concentrations of MnO₂ thereby increasing biological Mn(IV) reduction by the particle attached bacteria. The resulting higher concentrations of Mn(II) will then stimulate the activity of Mn(II)-oxidizing bacteria (thereby increasing the expression level of Mn(II)-oxidizing proteins) which will be reflected as higher Mn(II) oxidation rates in the plume during neap tides and the transition from neap to spring tide.

This simplified interpretation appears to hold true in the current study as cells concentrated from surface waters in the plume that were strongly influenced by the river end-member water (P1 and P2 ~ 4 days post neap tide) could produce between 7 and 10 fM MnO₂ cell⁻¹ day⁻¹ compared to only 1 to 2 fM MnO₂ cell⁻¹ day⁻¹ for (spring tide) samples that were heavily influenced by the coastal end member water (P3 and P4) (Fig. 3). This represents a 5 – 6 fold increase in Mn oxidation rates during the post neap tide period compared to spring tide, which is in close accordance with the 4-fold variability in Mn(II) reported by Aguilar-Islas and Bruland (2006). If only the heterotrophic manganese oxidizing cells are assessed

(using an average of the minimum and maximum 95 % confidence limits of manganese oxidizing bacterial numbers from the MPN data) then the consequent MnO_2 production is on the order of 0.04 to 92 $\text{nM cell}^{-1} \text{ day}^{-1}$ depending on the sample (Table 1). Although estimates for the Mn turnover in the estuary could not be properly ascertained due to interference from sediments, measurements of manganese oxidation by crude protein extracts (proteins separated from the sediment particles) after overnight incubation indicated that the estuary sample E1_12m proteins oxidized approximately 50 % more Mn^{2+} than ocean derived samples (data not shown).

For some samples there was an absence of Mn(II) oxidation in the MPN analysis but active *in situ* Mn(II) oxidation. This difference is probably due to heterotrophic Mn(II)-oxidizing bacteria being outcompeted by faster growing community members or inactivity in the conditions used in the MPN analysis. It is also possible that non-heterotrophic members of the bacterial community performing the Mn(II) oxidation process *in situ* (which cannot be assessed by the MPN assay) although autotrophic Mn(II) oxidation has not been previously demonstrated. Although we have no information about the autotrophic Mn(II)-oxidizing communities, the data suggests that the enzymes responsible for Mn(II) oxidation are extremely efficient since an *in situ* Mn-oxidizing signature can be detected from only a small proportion of the total heterotrophic bacteria.

When culturing Mn(II)-oxidizing bacteria, K and M plates supported the growth of the largest number of Mn(II)-oxidizing colonies (identified by brown precipitates and positive LBB spot tests). These isolates were then sub-cultured to ensure that they were pure. From the colonies sub-cultured only 2 distinct morphologies were observed. The DNA was extracted from these isolates and the SSU rRNA gene was amplified using PCR. The resulting PCR products were sequenced and then identified using a BLASTn search (NCBI database) as being from the genera *Shewanella* and *Bacillus*. Both these genera have been implicated in Mn redox cycling before with enzymes from the exosporium of *Bacillus* spores being responsible for Mn(II) oxidation (Dick *et al.*, 2008) and *Shewanella* being known as an oxidizer and reducer of various metals including manganese (Nealson and Myers, 1992; Nealson and Saffarini, 1994). The restriction cutting sites for the same enzymes used in the T-RFLP digest were located in the SSU rRNA sequences from *Bacillus* and *Shewanella* and potential peak signatures were found in the T-RFLP profiles at appropriate locations within ± 2 base pairs (Fig. 4). The same procedure was performed on SSU rRNA sequences from the Mn-oxidizing genera *Aurantimonas* (*Aurantimonas litoralis* was previously isolated off the Oregon coast, see Anderson *et al.*, 2009a) and *Rhodobacter* (Bräuer *et al.*, 2010). Potential peak signatures for both these genera were also found in the T-RFLP profiles to within ± 2 base pairs (Fig. 4).

Manganese oxidation and the proteins responsible

Five different sub-cellular protein fractions were recovered from the biomass concentrated with tangential flow filtration (TFF): secreted (proteins from the seawater at *in situ* concentration), loosely bound outer membrane (LBOM), soluble (periplasmic and cytoplasmic), inner membrane (IM) and outer membrane (OM). Each fraction was assayed overnight for Mn(II) oxidation activity where each sample was distributed into 2 sets of

triplicate wells in a 96-well plate and 100 μM MnCl_2 was added to one triplicate set while the other acted as a control. After incubation to allow enzymatic Mn(II) oxidation, LBB was added to all wells, and a measurement of the absorbance at 620nm was recorded. Only the LBOM and OM fractions had statistically relevant differences in absorbance with E1_12m, P1_2m and P3_2m showing the greatest Mn(II) -oxidizing potential (data not shown). This result is consistent with previous studies that have localized the manganese oxidation activity to the outer membrane and excreted protein fractions (Johnson and Tebo, 2008; Anderson *et al.*, 2009b) The proteins from each LBOM and OM fraction were separated using size exclusion chromatography and were subjected to another activity assay to identify which OD₂₈₀ absorbance peak(s) in the chromatography profile were associated with Mn(II) oxidation activity. The LBOM and OM fractions from E1_12m were the only post-FPLC samples with Mn oxidase activity. This activity was associated with fractions that eluted in one peak that came out of the column at approximately 76 minutes. Every sample had the same 76-minute peak so the active eluted fractions from E1_12m were used as a proxy for where the activity should be in the other samples. The active eluted fractions from each chromatography run were pooled together and concentrated for tandem mass spectrometry (MS/MS) analysis. MS/MS analysis was performed on the LBOM fraction from every sample and the OM fraction for samples E1_12m, P1_2m and P3_2m.

A total of 105 protein homologs were identified in GenBank with annotated function, with 84 % of these proteins returning homology to the extremely well annotated draft genome sequence from the marine Mn(II) -oxidizing alpha-Proteobacterium *Aurantimonas manganoxydans* sp. SI85-9A1. Other genera from which proteins were identified included: *Agrobacterium*, *Pseudomonas*, the alpha-Proteobacterium HTCC2255, *Ralstonia*, *Roseovarius*, *Pedobacter*, *Fulvimarina*, *Sinorhizobium*, and the algal genera *Gomphonema*, *Odontella*, *Thalassiosira* and *Encyonema*. From these, the only other genera apart from *Aurantimonas* that are known to oxidize manganese are *Pseudomonas* and *Fulvimarina* (Tebo *et al.*, 2004; Anderson *et al.*, 2009a).

The protein results are summarized in Table 2. The possible Mn(II) -oxidizing associated proteins included a putative hemolysin type Ca-binding heme peroxidase, a possible hemolysin type Ca-binding region, a putative multicopper oxidase (MCO), a quinone oxidoreductase and Fe/Mn superoxide dismutase (all identified with a probability of >99%). Motility proteins included flaggellin(s), flagellar hook associated and flagellar basal body associated. Transport and receptor proteins included amino acid transport proteins, tripartite ATP-independent periplasmic (TRAP) proteins, ABC transporters and substrate binding proteins, periplasmic substrate binding proteins, ligand receptors, signal peptides, solute binding proteins, a urea/thiourea/hydroxyurea transporter and porins. The enzymes identified included dehydrogenases, reductases, phosphatases, a carrier protein and transferases. One photosystem II protein was identified along with ATP synthase and cytochrome c554. The RuBisCO large subunit and RuBisCO small chain proteins were identified along with a nitrogen regulatory protein. Other proteins identified included basic membrane proteins, cold shock proteins, and 6 hypothetical proteins. Proteins isolated that should not have been associated with the LBOM fractions or the OM fractions (because they are believed to reside intracellularly) included a DNA binding protein, translation elongation factors, a ribosome recycling factor, chaperonin groEL, and a ribosomal protein.

From the manganese oxidizing associated proteins, the Ca-binding heme peroxidase (MopA, accession number: ZP_01225898) has recently been directly implicated with Mn(II) oxidation in *Aurantimonas manganoxydans* strain SI85-9A1 (Anderson *et al.*, 2009b). Peroxidase enzymes are also known in the fungal world to be involved in Mn(II) oxidation where they oxidize Mn(II) to Mn(III) which is then complexed and used to oxidatively degrade recalcitrant organic matter such as lignin (Wariishi *et al.*, 1992; Höfer and Schlosser, 1999; Schlosser and Höfer, 2002; Miyata *et al.*, 2007). MCO's have been identified as being important for Mn(II) oxidation in *Leptothrix*, *Pseudomonas*, *Pedomicrobium* and *Bacillus* (summarized by Ridge *et al.*, 2007). In the Mn(II) oxidizing strain *Erythrobacter* sp. strain SD21; the quinone PQQ has been reported as being important (Johnson and Tebo, 2008) suggesting that the quinone oxidoreductase identified may have a role. Many superoxide dismutase enzymes contain Mn in the reactive center enabling cells to adapt to oxidative stress.

The prevalence of *Aurantimonas* proteins and in particular the Ca-binding heme peroxidase, prompted a survey of the genomic DNA extracted from the same samples and estuarine samples collected in the summer of the previous year (2007). Q-PCR primers were designed for the *Aurantimonas* SSU rRNA gene using an alignment of eight *Aurantimonas* SSU rRNA genes and for the peroxidase using 17 orthologous heme-peroxidase genes. The NCBI database was then queried for these primer sequences using a BLASTn search with the SSU rRNA primers only returning hits to *Aurantimonas* sequence and the peroxidase primers only returning hits to homologous heme-peroxidases. Comparisons of the number of *Aurantimonas* SSU rRNA sequences to the total number of bacterial SSU rRNA sequences gave a figure of 0.040 – 0.045 % (1 in 2238 to 2513) for estuary samples (including samples collected in the previous year) (Fig. 5), 0.005 – 0.031 % (1 in 3182 to 20783) for plume samples (Fig. 5), and 0.002 % (1 in 48903) for surface offshore samples (data not shown).

The heme-peroxidase copy number in each sample was between 3.7×10^4 and 2.6×10^5 copies in the estuary (depending on the year – 2007 and 2008 respectively), 1.6×10^3 to 5.9×10^3 in the plume samples (Fig. 6) and 1.1×10^3 in surface offshore samples (data not shown). If one compares these numbers with the number of *Aurantimonas* (using an average of three SSU rRNA gene copies per cell and one heme-peroxidase copy per cell) then *Aurantimonas* can only account for a maximum of 1.7 % of the heme-peroxidase quantified from the environment. From Table 3, orthologs of the *Aurantimonas manganoxydans* heme-peroxidase are found in 11 different sequenced genomes, suggesting greater gene diversity is found in environmental samples. The proportion of these heme-peroxidases that actually oxidize Mn is still unknown, but the potential exists for the process of peroxidase-driven Mn(II) oxidation to be very common in the environment.

Two important observations have been made throughout this study; 1) active bacterial Mn(II) oxidation is detectable within the microbial populations of the Columbia River Estuary and Plume, and 2) the proteins responsible can be isolated and identified from community sub-cellular protein fractions. The quality of protein data from environmental organisms in the databases is now at a level where positive identifications of environmental proteins can be made. From laboratory studies we know that heme peroxidases and multicopper oxidases

(MCO's) are required for Mn(II) oxidation and now the same proteins have been identified *in situ*.

Combining the culture work, T-RFLP data and the *in situ* protein data we have strong evidence to link the biogeochemical cycling of manganese in the Columbia River estuary and the offshore plume with species from the genera *Aurantimonas*, *Bacillus*, *Shewanella* and *Rhodobacter*. The T-RFLP community fingerprinting shows a strong correlation between salinity and overall community structure. The combined data also closely connects estuarine microbial assemblages to plume assemblages supporting active transport of bacterial Mn redox cycling capability from the estuarine environment to the coast and offshore. Proteins from *Pseudomonas* and *Fulvamarina* were also identified and both these organisms have been implicated in Mn(II)-oxidizing bacteria are present and taking part in active manganese biogeochemical cycling in the Columbia River and plume is therefore plausible and supports the protein data where multiple genera are represented. There is strong evidence to suggest that Mn(II) oxidation in the Columbia River and Plume is driven by bacterial heme peroxidases and potentially MCO's from multiple genera.

It is difficult to gauge the actual amount of Mn(II) oxidised as estimates approaching 100 nM cell⁻¹ day⁻¹ (MPN assay) vastly overestimate the rate as the assay promotes the growth of fast growing heterotrophic Mn(II)-oxidizers whereas the fM cell⁻¹ day⁻¹ figures (*in situ* whole cell Mn(II) oxidation assay) reflect non-optimised assays with questionable cell viability after sample handling and storage. The conditions used for the *in situ* assay also reflect conditions used for heme-peroxidase proteins extracted from pure cultures of *Aurantimonas manganosydans* strain SI85-9A1 and will be far removed from the conditions needed to gauge oxidation rate properly in an *in situ* setting.

In situ measurements of ⁵⁴Mn²⁺ removal have been recorded between 2 to 50 nM hr⁻¹ for hydrothermal vents in the Guaymas Basin and the Black Sea but the number of cells capable of Mn(II) oxidation in these studies is unknown (Dick *et al.*, 2009; Clements *et al.*, 2009). If these rates can be extended to the Columbia River estuary and plume, then 3.6 to 90 hours would be required to produce the 180 nM difference in dissolved manganese concentrations observed between spring and neap tides (Aguilar-Islas and Bruland, 2006). It is therefore entirely feasible that the bacterial community in the Columbia River and plume can achieve similar oxidation rates making this system an extremely significant biogeochemical process supporting primary productivity (through recalcitrant carbon release and trace-metal transport) vast distances along the NW coast of America.

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Figure 1.

Sample locality map of the Columbia River, estuary, Oregon coast and offshore where sampling depths are indicated alongside sample type identifier (E=Estuary, P=Plume, C=Coastal and O=Ocean). Samples indicated in bold and italics font represent 60 L volume samples for both DNA and protein extraction whereas samples represented in regular font were 10 L volume samples for DNA extraction only. Samples O1 and C1 were selected as control samples as they had less influence from the Columbia River. Samples P1, P2, C1, C2 and C3 were taken on June 1st 2008, 4 days after neap tide whereas samples P3 and P4 were taken on the 3rd June 2008, during the spring tidal cycle. E1 and E2 were taken on June 2nd 2008.

Figure 2.

Cluster analysis of bacterial communities in relation to practical salinity units (PSU). Panel (A) represents a hindcast of the salinity at the surface on June 1st 2008 at 5pm. Note the extreme freshening of coastal waters as the Columbia River water discharges. Panel (B) represents a hindcast of salinity at depth during the spring tidal cycle on June 2nd 2008 at midday. Note the saltwater incursion into the estuary. Panel (C) represents a UPGMA/Pearson product moment correlation cluster analysis of T-RFLP bacterial community fingerprints from all the samples collected. Scale bar is the Pearson product moment correlation r -value. Numbers at the nodes are cophenetic correlation coefficients. The bacterial community represented at sites P1_2m and E1_12m are river derived and indicate a transfer of microorganisms from the river/estuary to offshore whereas microorganisms from

the coast (more saline) are influencing the community at E2_11m. The near surface plume environment is heavily influenced by deep coastal microorganisms suggesting mixing of deep and surface waters while organisms from the near surface coastal environment north and south of the river mouth and offshore samples form a distinctive cluster on their own.

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Figure 3.

In situ whole cell Mn(II) oxidation activity normalized to total bacterial cell number. The total bacterial cell numbers were calculated from Q-PCR data using an average of three SSU rRNA gene copies per cell. The figure representing E1_12m is not accurate due to sediment interference in the LBB assay.

Figure 4.

A1uI T-RFLP electropherograms of the Bacterial communities from the river and plume samples E1_12m and P1_2m respectively. Arrows indicate the predicted terminal restriction fragment for the genus *Aurantimonas* and Mn-oxidizing bacteria isolated from the Columbia River.

Figure 5.

Percent *Aurantimonas* SSU rRNA copies in proportion to total Bacterial SSU rRNA gene copies amplified by Q-PCR from the same genomic DNA extracts. The 2007 samples were collected during the summer in the Columbia River Estuary. ETM_In was collected during an estuarine turbidity maximum (ETM) event and ETM_Out was collected after the ETM had passed.

Figure 6.

Copies of the *Aurantimonas* manganese oxidizing peroxidase gene (*mopA*) and homologs compared to the numbers of *Aurantimonas manganoxydans* SSU rRNA gene copies amplified by Q-PCR from the same genomic DNA extracts. The 2007 samples were collected during the summer within the Columbia River Estuary. ETM_In was collected during an estuarine turbidity maximum (ETM) event and ETM_Out was collected after the ETM had passed.

Table 1

Summary table of bacterial numbers (per mL) present in the Columbia River Estuary and plume environments and their ability to oxidize manganese. MPN data only represents culturable heterotrophic bacteria (HB) and includes the upper and lower 95 % confidence interval (CI) limits. The total bacterial numbers were calculated from Q-PCR data using an average of three SSU rRNA gene copies per cell. Figures for MnO₂ generation were calculated based on the concentration of MnO₂ produced over a 48-hour period and an average of the 95% CI MPN figures for Mn oxidizing heterotrophic bacteria (MOHB). It does not account for any Mn oxidation performed by bacteria from other metabolic groups.

Sample	Heterotrophic bacteria (HB)				Mn(II)-oxidizing Heterotrophic Bacteria (MOHB)				Total Bacteria (Q-PCR assuming three SSU rRNA gene copies cell ⁻¹)	% Culturable Bacteria	% Culturable Mn(II)-oxidizing Bacteria	nM MnO ₂ cell ⁻¹ day ⁻¹ (Ave. 95% CI MOHB MPN)	
	95% CI lower		MPN (mL) ⁻¹	95% CI upper	95% CI lower	MPN (mL) ⁻¹	95% CI upper	Maximum				Minimum	
P1_2m	1.0 × 10 ³	2.4 × 10 ³	5.9 × 10 ³	-	-	-	-	3.5 × 10 ⁴	6.8	-	61.0	7.5	
P2_19m	4.7 × 10 ²	1.2 × 10 ³	3.0 × 10 ³	8.0 × 10 ⁻²	3.0 × 10 ⁻¹	1.2 × 10 ⁰	-	4.7 × 10 ⁴	2.5	0.0006	92.1	11.3	
P3_2m	1.2 × 10 ⁴	3.1 × 10 ⁴	7.7 × 10 ⁴	-	-	-	-	9.8 × 10 ⁴	31.6	-	15.7	1.9	
P4_102m	4.6 × 10 ⁴	1.2 × 10 ⁵	2.9 × 10 ⁵	8.0 × 10 ⁻²	3.0 × 10 ⁻¹	1.2 × 10 ⁰	-	8.9 × 10 ⁴	1.4	0.0003	5.0	0.6	
E1_12m	1.0 × 10 ⁴	2.5 × 10 ⁴	6.2 × 10 ⁴	2.6 × 10 ⁰	7.3 × 10 ⁰	2.1 × 10 ¹	-	3.1 × 10 ⁶	0.8	0.0002	0.3 [*]	0.04 [*]	

* The figures calculated for E1_12m are not accurate due to sediment interference in the LBB assay.

Number of proteins identified with a probability of >90% grouped by potential function from the loosely bound outer membrane (LBOM) and outer membrane (OM) protein fractions. Numbers in parenthesis are additional identifications where the probability of correct identification is below 90%.

Table 2

Proteins Identified	Loosely Bound Outer Membrane Proteins					Outer Membrane Proteins		
	P1_2m	P2_19m	P3_2m	P4_102m	E1_12m	P1_2m	P3_2m	E1_12m
Mn Oxidizing Associated	1	1	-	-	5	1	1	1
Motility	4 (4)	8 (2)	-	1	18 (1)	2 (6)	3 (6)	6 (2)
Transport and Receptors		4 (2)	1 (2)	4 (2)	22	4	2	(1)
Enzymes	2	2 (2)	1 (1)	1	21	1 (1)	1	1 (1)
Cytochromes/ Photosystem/ ATP Generation	-	-	-	-	2	1	1	(1)
Carbon Fixation and Nitrogen	-	-	-	-	-	1	3	(1)
DNA binding/ Transcription/ Translation	-	-	-	-	9	1	1	-
Miscellaneous / Hypothetical	-	-	-	-	11	(1)	-	(1)

Table 3

Homologs to the *Aurantimonas manganoxydans* Ca-binding heme peroxidase (MopA, Accession number: ZP_01225898). Organisms in bold font include species and genera known to oxidize manganese.

Sequences producing significant alignments		Organism	Score (Bits)	E Value
ZP_01438926.1	Hemolysin-type calcium binding bacteriocin	<i>Fulvimarina pelagi</i> HTCC2506	2202	0.0
YP_001639132.1	Heme peroxidase	<i>Methylobacterium extorquens</i> PA1	1886	0.0
ZP_02054919.1	Animal haem peroxidase	<i>Methylobacterium chloromethanicum</i> CM4	1885	0.0
YP_568698.1	Heme peroxidase	<i>Rhodopseudomonas palustris</i> BisB5	1067	0.0
ZP_01880359.1	Animal haem peroxidase	<i>Roseovarius</i> sp. TM1035	1062	0.0
ZP_01865380.1	Animal haem peroxidase	<i>Erythrobacter</i> sp. SD-21	1033	0.0
YP_673356.1	Heme peroxidase	<i>Mesorhizobium</i> sp. BNC1	944	0.0
ZP_01058523.1	Hemolysin-type calcium binding bacteriocin	<i>Roseobacter</i> sp. MED193	916	0.0
NP_744706.1	Hemolysin-type calcium binding bacteriocin	<i>Pseudomonas putida</i> KT2440	867	0.0
YP_001669581.1	Animal haem peroxidase	<i>Pseudomonas putida</i> GB1	847	0.0
ZP_01904565.1	Animal haem peroxidase	<i>Roseobacter</i> sp. Azwk-3b	801	0.0
YP_001791329.1	Heme peroxidase	<i>Leptothrix cholodnii</i> SP-6	762	0.0
YP_830737.1	Heme peroxidase	<i>Arthrobacter</i> sp. FB24	734	0.0