environmental microbiology reports

Environmental Microbiology Reports (2011) **3**(6), 682–688 doi:10.1111/j.1758-2229.2011.00281.x

Possible influence of bacterial quorum sensing on the hydrolysis of sinking particulate organic carbon in marine environments

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Summary

A central component of the ocean's biological carbon pump is the export of sinking, photosynthetically derived, particulate organic carbon (POC). Bacteria colonize these particles and produce enzymes that hydrolyse sinking POC thereby acting as one of the major controls on the biological pump. Here we provide evidence that a bacterial cell–cell communication mechanism, quorum sensing (QS), may influence the activity of hydrolytic enzymes on sinking particles. We collected sinking POC from a site off Vancouver Island, Canada and found that it contained acylated homoserine lactones (AHLs), a suite of well-known bacterial communication molecules. Furthermore, we observed that the addition of exogenous AHLs to incubations containing sinking POC affected the activity of key hydrolytic enzymes involved in POC degradation in some cases. Our results suggest that AHL-based QS could play an important role in regulating the degradation of sinking POC and that variability in AHL-triggered POC hydrolysis is a heretofore unrecognized process that impacts the marine biological carbon pump.

Introduction

Carbon fixed by photosynthesis is transferred to the deep ocean via sinking particulate organic carbon (POC) where it is removed from the atmosphere for hundreds to thousands of years. Sinking POC flux declines significantly in the upper few hundred meters of the ocean (Martin *et al*., 1987; Buesseler *et al*., 2008), but there is significant spatial and temporal variability in this attenuation (Buesseler *et al*., 2007). It is generally accepted that POC-attached bacteria contribute significantly to organic carbon degradation during sinking (Steinberg *et al*., 2008), but parameterizing their contribution remains a major obstacle to the development of accurate carbon cycle models. Currently, flux attenuation is represented by empirical (Martin *et al*., 1987) or chemical models (Armstrong *et al*., 2001), which do not explicitly incorporate the activities of particle-attached bacteria.

Many bacteria use a cell-density dependent signalling system, 'quorum sensing' (QS) to coordinate the expression of genes encoding behaviours that benefit cells at high population density (e.g. extracellular hydrolytic enzyme production, luminescence, biofilm formation). Extracellular hydrolytic enzymes are used by particle-attached bacteria in the conversion of sinking POC to suspended POC or dissolved organic carbon (DOC) in the environment (e.g. Smith *et al*., 1992). Bacteria that produce the particular class of QS signal molecules, acylated homoserine lactones (AHLs), are readily isolated from marine environments where bacterial population densities are high (Gram *et al*., 2002; Wagner-Dobler *et al*., 2005). At this time there are only a limited number of cultivation-independent reports of AHLs from natural marine environments (e.g. Decho *et al*., 2009).

Although micromolar concentrations of AHLs have been reported in bacterial cultures (Boettcher and Ruby, 1995; e.g. Puskas *et al*., 1997), many bacteria can respond to concentrations in the nanomolar range (Kaplan and Greenberg, 1985; Boettcher and Ruby, 1995; Burton *et al*., 2005). In the marine environment, AHLs are most likely to accumulate in microenvironments where bacterial densities are high (Hmelo and Van Mooy, 2009), such as within sinking POC. Direct measurements of AHLs in environmental samples require extremely sensitive and selective analytical tools, which are only now becoming available to the oceanographic community. Although bacterial cultivars from marine particles have been shown to possess the capacity for QS (Gram *et al*., 2002), *in situ* QS activity has yet to be demonstrated in sinking POC. In this study, we investigate the occurrence of QS in sinking POC, test for linkages between QS and

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hydrolytic enzyme activity, and consider the potential impact of QS-regulated POC hydrolysis on the flux of sinking POC in the oceans.

Results and discussion

In order to test the hypothesis that AHL-QS occurs on marine particles, we collected sinking POC during multiple deployments of sediment net traps (Peterson *et al*., 2005) in Clayoquot Sound on the western coast of Vancouver Island, B.C. in July 2009 aboard the R/V *Clifford A. Barnes.* We chose Clayoquot Sound because of its high fluxes of marine photosynthetically derived POC in the summer months. During our sampling, the euphotic zone was observed to extend no deeper than 30 m. Sinking POC was collected over the course of 24 h by traps suspended at 55 m on four separate days (Trap 1, 2, 3 and 4). The temperature at 55 m was 9°C. The recovered POC was assayed for AHLs by two independent methods. First, POC from the traps was applied to agar plates seeded with the AHL-sensing bacterium *Agrobacterium tumefaciens NTL4 (pZLR4)* (Farrand *et al*., 2002) while we were at sea. The POC induced the distinct response of this biosensor (Fig. S1), indicating that the particles contained compounds with the bioactivity of AHLs. Following an approach similar to Gram and colleagues (2002) we isolated bacteria from POC on agar plates. Of the 160 unique isolates we obtained, 21 tested positive with the *Agrobacterium* assay and 16S rRNA gene analysis showed that these isolates were representatives of common marine genera (Table S1).

Since biosensors can be subject to false positive results (Holden *et al*., 1999) and do not by themselves provide the identity of the specific AHL detected, a sample from Trap 4 was also collected and returned to the laboratory at Woods Hole for analysis by high performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS). We employed a triple quadrupole MS and structurally identified two AHLs, *N*-(octanoyl) homoserine lactone (C8-HSL) and *N*-(dodecanoyl) homoserine lactone (C12-HSL), on the basis of their chromatographic retention time, molecular masses and secondary ion mass spectra, which were identical to authentic standards (Fig. 1). Using a Fourier-transform ion cyclotron resonance MS, we additionally identified *N*-(3-oxohexanoyl) homoserine lactone (3-oxo-C6-HSL) and *N*-(3-oxooctanoyl) homoserine lactone (3-oxo-C8-HSL; Fig. S2). All process blanks were devoid of AHLs. The 3-oxo-C6-HSL and 3-oxo-C8-HSL were less abundant than the C8- and C12-HSL, which might reflect the shorter residence time of 3-oxo AHLs in seawater (Hmelo and Van Mooy, 2009). Overall, these mass spectrometry results confirm that AHLs were present in sinking POC samples and were likely responsible for the biosensor response. Since AHLs are known to have a rapid turnover time in natural seawater (0.5–1.5 day^{-1} depending on the structure) (Hmelo and Van Mooy, 2009), the detection of these compounds in our POC samples is indicative of *in-situ* QS activity. This study represents the first time that AHLs have been detected in sinking POC.

Smith and colleagues (1992; 1995) have demonstrated that particle-attached bacteria are responsible for intense hydrolytic enzyme activity on sinking POC (but see Huston and Deming, 2002). It has been suggested by Vetter and colleagues (1998) that coordination of extracellular hydrolytic enzyme production is advantageous for POC-attached bacteria, and that coordinated action by microbial consortia or clonal populations would maximize the energetic returns to an individual bacterium on the release of enzymes to the environment. Our observation of AHLs in sinking POC is consistent with such coordinated behaviour. During our cruise we conducted incubation experiments to test whether AHL-QS affected rates of organic carbon hydrolysis.

We quantitatively split the sinking POC samples retrieved from the traps into sets of triplicate incubations, which were then amended with either 3-oxo-C6-HSL or 3-oxo-C8-HSL. We chose these two AHLs because they are produced by *Vibrio fischeri* (Kuo *et al*., 1994), the marine bacterium in which QS was first described. As described above, these two AHLs were detected in sinking POC. Although C8-HSL and C12-HSL were also detected, we had no foreknowledge of which AHLs would be present in our samples of sinking POC (conducting HPLC/ESI-MS analyses of AHLs on the R/V *Clifford A. Barnes* was not possible), thus we were not able to adjust our experimental design to include C8-HSL and C12-HSL amendments. The initial concentrations of both 3-oxo-C6-HSL and 3-oxo-C8-HSL in the incubations were 500 nM. Based on the measured degradation rates of 3-oxo-C6-HSL and 3-oxo-C8-HSL in natural coastal seawater (Hmelo and Van Mooy, 2009), only 6% and 3%, respectively, of the two AHL amendments were expected to be bioactive after 24 h (expected AHL loss due to abiotic lactonolysis and enzymatic degradation). Therefore, we also conducted incubations with a starting AHL concentration of 5000 nM in order to ensure at least 100 nM AHL at the conclusion of the 24 h incubation experiments. Thus, the initial and final concentrations of AHLs in these experiments spanned the range of concentrations observed in pure cultures of AHL-producing bacteria (Boettcher and Ruby, 1995; Puskas *et al*., 1997).

At two time points (6 and 24 h) we measured aminopeptidase, lipase, phosphatase, chitobiase, and α- and β-glucosidase activity in the incubations using model fluorogenic substrates (Hoppe, 1993); this same type of approach was applied by Smith and colleagues (1992) in their study. We then compared the incubations amended

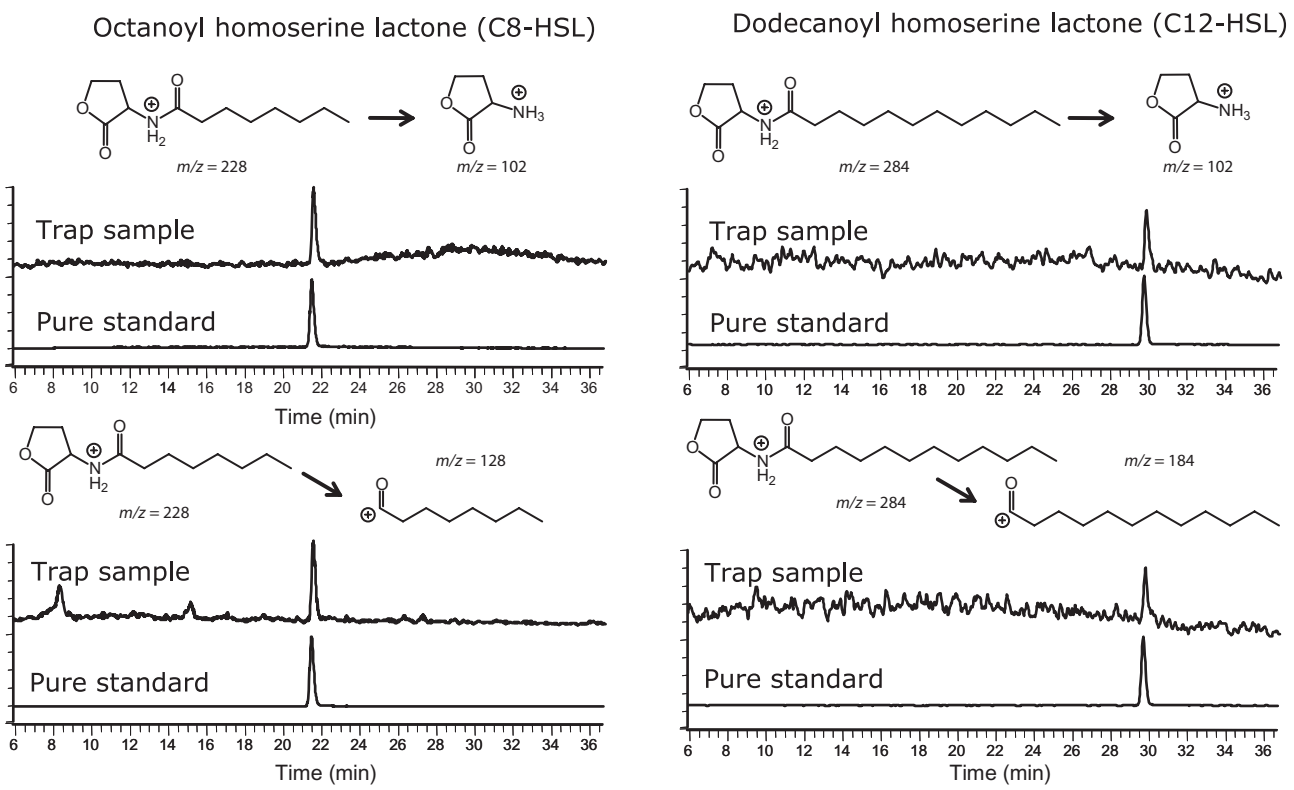

Fig. 1. HPLC/ESI-MS data showing the presence of AHLs in POC from Trap 4. Each chromatogram records the intensity of the diagnostic AHL fragmentation as detected by our MS. The left quadrants show the fragmentation of the C8-HSL and the right quadrants show the fragmentation of the C12-HSL. The upper quadrants show the intensity of diagnostic lactone ring fragment (m/z 102) from the respective molecular ions of C8-HSL and C12-HSL, while the lower quadrants show the intensity of the acyl chain fragments. In each quadrant, upper chromatograms are from our sinking POC samples and lower traces are from authentic standards. Samples were collected using moored sediment net traps (Peterson *et al*., 2005) in Clayoquot Sound (49°9.93′N, 125°41.56′W) off the western coast of Vancouver Island at 55 m depth. Traps were deployed for 24 h on separate days (Traps 1,2, 3 and 4) in June 2009. Fine-mesh windows (50 μm) in the cod end of the traps allow exchange of seawater during the trap deployment, and thus prevented anoxic conditions from developing. Samples of sinking POC from Trap 4 were designated for analysis by mass spectrometry. These samples were acidified to pH 2 and frozen at –20°C and transported back to Woods Hole where they were thawed and extracted in ethyl acetate containing 0.1% formic acid according to previously published methods (Hmelo & Van Mooy, 2009). Extracts were analysed by using HPLC coupled to a Thermo Vantage-TSQ triple quadrupole mass spectrometer in selected reaction monitoring (SRM) mode or a Thermo LTQ-Ultra ion trap/ 7-T Fourier transform-ion cyclotron resonance MS. The reversed-phase chromatography methods we employed have been previously described (Hmelo & Van Mooy, 2009). All procedural blanks were free of detectable AHLs.

with AHLs to controls without AHLs and found clear evidence that AHLs enhanced the level of enzyme activity in some instances (Fig. 2). We observed a strong overall response to AHL amendments with particles collected during our first trap deployment (Trap 1; Fig. 2): aminopeptidase (Kruskal–Wallis ANOVA; $P = 0.012$) activity was enhanced by AHL treatments by as much as a factor of two, while lipase ($P = 0.008$) and phosphatase ($P = 0.002$) activity were enhanced by as much as factors of 3 and 8 respectively (Fig. 2). However, aminopeptidase activity ($P = 0.018$) was markedly enhanced primarily by 3-oxo-C8-HSL in Trap 2, and not by any AHLs in Trap 3 incubations (Fig. 2). Lipase activity was not affected by AHLs in Trap 2 incubations, but in Trap 3 incubations ($P = 0.164$) appeared to be enhanced by 500 nM amendments of 3-oxo-C6- and 3-oxo-C8-HSL after 6 h, although this was not found to be statistically robust. Phosphatase activity was impacted by AHLs in Trap 2 incubations ($P = 0.035$) and in Trap 3 incubations ($P = 0.048$), with 3-oxo-C8-HSL generally having the greatest effect. By comparison, the three glucosidases (chitobiase, α-glucosidase and β-glucosidase) were not affected ($P > 0.05$) in all three traps (Fig. S3). Over the course of the trap deployments, we observed a succession in the zooplankton that were unintentionally collected in the traps; we speculate that these organisms may have affected the trap-to-trap variability in our data. Altogether, these results indicate a biochemical linkage between AHLs and enzyme activity that may be variable in time and co-regulated by environmental factors such as food web structure, particle properties and microbial community composition.

The observation that AHLs enhanced the activity of key hydrolytic enzymes supports the hypothesis that

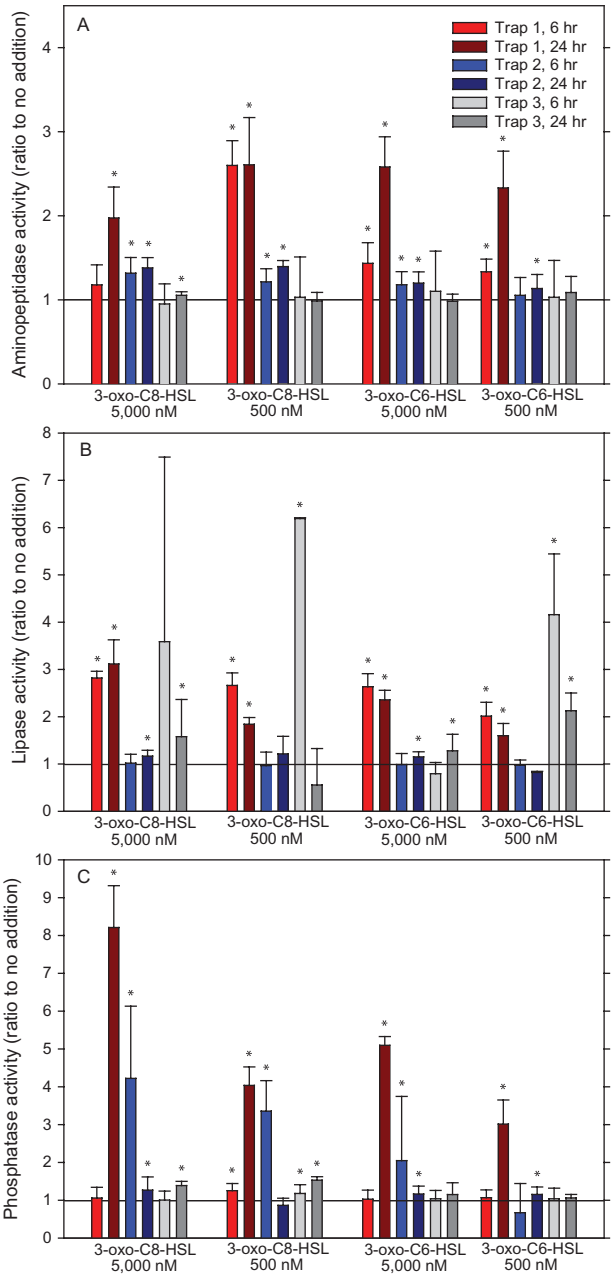

Fig. 2. Changes in aminopeptidase (A), lipase (B) and phosphatase (C) hydrolytic enzyme activity in response to amendments of 3-oxo-C6-HSL and 3-oxo-C8-HSL. The *y*-axis presents the ratios of experimental rates relative to unamended-control rates, where a value of 1 reflects no response to AHLs. Error bars represent the magnitude of the range of the data, and asterisks denote significant ($P < 0.05$) difference rates from controls (i.e. a value of 1) by Mann–Whitney test conducted with Statistica (Statsoft) statistical software (note: Mann–Whitney test does not account for multiple comparisons, please refer to Kruskal–Wallis test results in text). Note, the *y*-axis scale varies in (A), (B) and (C). POC samples were collected for use in enzyme assays from Traps 1, 2 and 3. Large zooplankton were removed by pouring the samples collected in the traps through a sterile fabric screen into a sterile container. POC samples were quantitatively split into equal volumes using a Folsom-style splitter (Aquatic Research Instruments; Van Guelpen *et al.*, 1982) and placed in centrifuge tubes. Particles were gently rinsed as follows: each aliquot was centrifuged for 1 min at 3000 r.p.m. and the supernatant was poured off. Next, 10 ml of 0.2 μm filter-sterilized seawater from 55 m was reintroduced to the particles. Particles were gently stirred in a temperature controlled shaking incubator, centrifuged and the supernatant was poured off. This procedure was followed one additional time (three washes total), returning the samples to the incubator between treatments to maintain *in situ* temperature (9°C). Finally, particles were resuspended in 10 ml of 0.2-μm-filtered seawater by gently stirring. We amended triplicate tubes with either 500 nM or 5000 nM of 3-oxo-C6-HSL or 3-oxo-C8-HSL (Sigma-Aldrich). We conducted triplicate control incubations that contained particles, but no AHL amendments. The incubations were continuously stirred in the dark at 9°C and sampled at 6 and 24 h intervals. At each sampling time point, an aliquot of the incubation was removed and assayed for one of six enzyme activities. Enzyme activities were assayed by observing the hydrolysis product of the commonly used fluorogenic substrates (all from Sigma-Aldrich): L-leucine-7-amino-4-methylcoumarin (MCA-leucine; for aminopeptidase activity), 4-methylumbelliferyl (MUF)-phosphate (for phosphatase activity), MUF-*N*-acetyl-β-D-glucosamine (for chitobiase activity), MUF-butyrate (for lipase activity), MUF-α-glucopyranoside and MUF-β-glucopyranoside (for α- and β-glucosidase activity respectively) (Smith *et al.*, 1992; Hoppe, 1993). Aliquots of the AHL incubation were placed in 96-well plates, fluorescent substrates at saturating concentration (50 μmol l^{-1}) were added to initiate the assays, and the plates incubated at *in situ* temperature for approximately 2 h. Fluorescence was measured at regular intervals with a CytoFluor Series 4000 multi-well plate reader, and was found to increase linearly with time. Using standard MUF and MCA additions, and an external standard curve, fluorescence readings were converted to concentration units. Enzyme hydrolysis rates were calculated from the linear increase in MUF and MCA concentrations over time during the assays.

AHL-based QS could contribute to variability in the hydrolysis of sinking POC. However, an alternative explanation deserves consideration: instead of acting as signalling molecules, AHLs could have provided a carbon and/or nitrogen supplement to particle-attached bacteria and thereby the observed changes in enzyme activity could have reflected changes in bacterial nutrition. We discount this latter explanation for the following reasons: first, the amount of carbon we added as AHL was likely to have been orders of magnitude lower than the DOC concentration in the interstitial water (~ 1–5 mM) of typical marine aggregates (Alldredge, 2000), and thus it is unlikely that the small amount of AHLs would stimulate a dramatic response; second, AHL amendments elicited a response in only a subset of the enzyme activities we examined during each set of incubations, which suggests regulation of specific behaviours as opposed to a general microbial response; third, additional AHL in the 5000 nM treatments did not systematically enhance the effect induced by the 500 nM treatment, which is suggestive of the threshold response characteristic of QS. In consideration of the preceding arguments, we believe that our data best support the first explanation: AHL-based QS is a factor in regulating the expression of hydrolytic enzymes. Clearly, future studies could take advantage of community

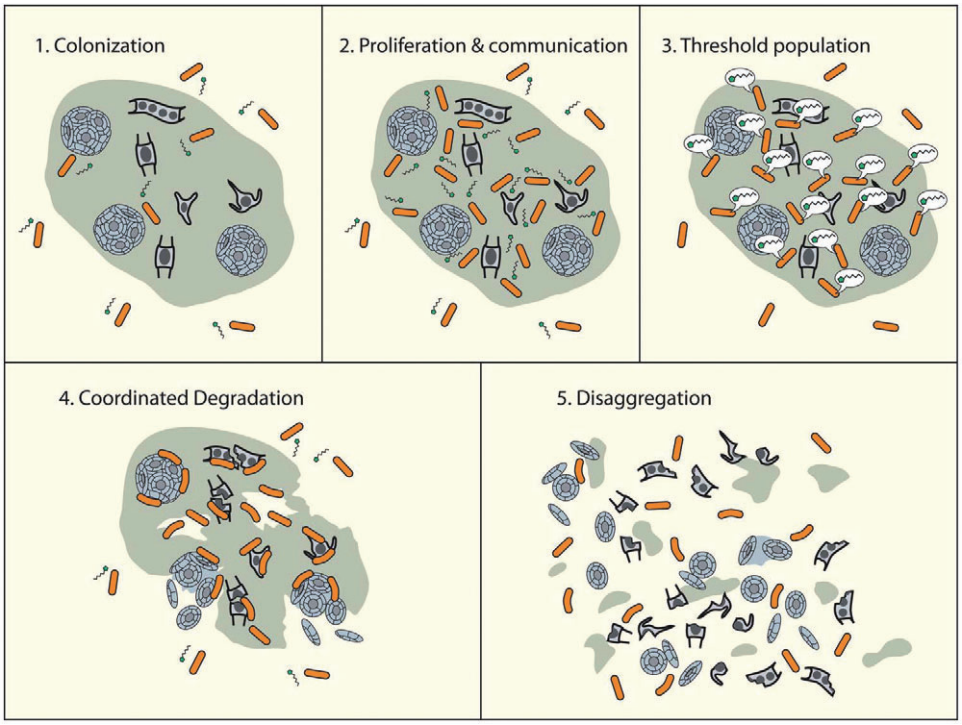

Fig. 3. Conceptual illustration of how QS-mediated increases in hydrolytic enzyme activity might impact a sinking POC particle through a succession of five main steps.
1. Bacteria colonize or are encapsulated in freshly formed sinking POC.
2. Bacteria produce QS signals at a basal rate while they proliferate and clonal populations grow.
3. A particular QS-signal concentration threshold signifies to bacteria that a threshold population has been reached.
4. At this population threshold, bacteria initiate a coordinated expression of hydrolytic enzymes.
5. The production of hydrolytic enzymes leads to the disaggregation of sinking POC into smaller suspended POC (or dissolution to DOC).

transcriptomic and proteomic techniques to examine the underlying regulation of hydrolytic enzyme activity by QS in sinking POC.

Model calculations have been performed to examine the potential for particle-attached bacteria to benefit from coordinated production of extracellular hydrolytic enzymes (Vetter *et al*., 1998). These models predict that hydrolysates produced by the enzymes from one bacterium could be lost through diffusion to the environment and surrounding cells, but if a group of cells are using QS to act in concert then a loss from one cell is a gain for the other. Our observations that hydrolytic enzyme activity is enhanced by exogenous AHLs in sinking particles provide support for this prediction, and we speculate that there are at least two important biogeochemical implications for this finding. First, by accelerating the solubilization of POC, POC-associated bacteria are retained in particle-rich surface waters to which they are adapted. Second, by preferentially transferring N- and P-containing biomolecules from the sinking POC to the suspended POC or DOC pool (via enhanced aminopeptidase, lipase and phosphatase activity), these same cooperative behaviours may in fact be promoting the retention of major nutrients in surface waters, which in turn could support additional primary productivity and bacterial production; this speculation echoes that of Smith and colleagues (1992) and Huston and Deming (2002).

If, as our data suggest, AHL-based QS imposes variability on the timing and rate of organic carbon hydrolysis in sinking particles (Fig. 3), then QS could be a factor in determining the remineralisation–depth distribution of sinking POC. A recent study has shown that modest changes in the remineralization depth can have a major impact on atmospheric carbon dioxide concentrations (Kwon *et al*., 2009), thus QS could be a critical link between atmospheric and oceanic reservoirs of carbon. Our results suggest that QS is a heretofore unexplored molecular mechanism that could impact the flux of sinking POC in the world's oceans. We suggest that this variability in organic matter hydrolysis caused by AHL-based QS may someday be incorporated into carbon cycle models to better capture spatial and temporal variability in POC flux. Additional work is required in order to identify the environmental and biological parameters (e.g. particle composition, bacterial cell density and bacterial community structure) that determine when and where QS might affect sinking POC hydrolysis rates.

Acknowledgements

We thank R. Keil for generously allowing us to use his sediment traps, providing invaluable field support, and offering his knowledge of Clayoquot Sound. We thank E. Kujawinski and M. Soule for assistance with Fourier-transform ion cyclotron resonance MS analyses at WHOI. We also thank K. Buesseler for his insightful comments on an earlier version of this manuscript. We are also grateful to J. Cook for his assistance in preparing Fig. 3, S. K. Farrand for providing the *Agrobacterium tumefaciens* strains, the captain and crew of the R/V *Clifford A. Barnes* for a successful field campaign, and R. Kantor for her fieldwork assistance. This work was funded by a grant from the National Science Foundation to B.V.M and T.J.M. (OCE-0825407) and a grant from the Office of Naval Research to B.V.M. (N00014-09-1-0091).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Results of biosensor assays. Samples were applied in triplicate 2.5 μl aliquots in three rows and controls were applied beneath samples. Top row is particle-free seawater, middle row is diluted (~ 1:10) particulate matter, and bottom row is non-diluted particulate matter. Blue zones indicate AHL-like bioactivity of POC samples on agar plates seeded with the biosensor, bacterial strain *Agrobacterium tumefaciens NTL4(pZLR4)* and 40 μg ml^{-1} bromo-chloro-indolyl-galactopyranoside (X-Gal, 5 Prime, Hamburg, Germany), and control plates containing only 40 μg ml^{-1} X-Gal (Farrand *et al*., 2002). Large blue spot reflects where an aliquot of the positive control strain *A. tumefaciens NTL4(pTiC58)* was added. No spots were detected on the negative control *A. tumefaciens NTL4.*

Fig. S2. HPLC/FT-ICR-MS data showing endogenous 3-oxo-C6-HSL in sinking POC collected in Trap 4. All three chromatograms are extracted ion chromatograms displaying nominal m/z 214. Top and bottom chromatograms show retention of a standard 3-oxo-C6-HSL, run before and after an AHL extract from sinking POC as indicated by the middle chromatogram. Regrettably, the HPLC column had not completely equilibrated before these analyses and the standard 3-oxo-C6-HSL peak shifted by 0.28 min between the two standard runs. An m/z 214 peak elutes in this window in the sinking POC extract. The inset shows the FT-ICR-MS data at 4.71 min where a peak with m/z of 214.1074 was present; this is identical in mass to the 3-oxo-C6-HSL. The high mass resolution of the FT-ICR-MS allows 3-oxo-C6-HSL to be detected in the sinking POC extract despite the co-eluting m/z 214.1439. Blanks were run following both standards, and no carryover of the standard was detected.

Fig. S3. Results of α- and β-glucosidase activity assays presented as ratios of experimental rates to unamended-control rates. Error bars represent the magnitude of the range. Note that ratios are less than one in several Trap 3 incubations, which may indicate that chitinase activity was repressed by the AHL additions. Interestingly, a recent report indicates that chitinase activity in *Vibrio harveyi*, a common marine *Vibrio* species, is repressed when its QS genes are activated (Defoirdt *et al*., 2010).

Table S1. Identity of AHL-producing isolates from sinking POC.

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