Quorum sensing by N-acylhomoserine lactones is not required for *Aeromonas hydrophila* during growth with organic particles in lake water microcosms

Katharina Styp von Rekowski, Melanie Hempel, Bodo Philipp

Abstract It was investigated whether quorum sensing (QS) mediated by *N*-acylhomoserine lactones (AHLs) was important for heterotrophic bacteria from the littoral zone of the oligotrophic Lake Constance for growth with organic particles. More than 900 colonies from lake water microcosms with artificial organic aggregates consisting of autoclaved unicellular algae embedded in agarose beads were screened for AHL-production. AHL-producing bacteria of the genus *Aeromonas* enriched in the microcosms but AHLs could not be detected in any microcosm. To test for a potential function of AHL-mediated QS, growth experiments with the wild type and an AHL-deficient mutant of *Aeromonas hydrophila* in lake water microcosms were performed. Growth of both strains did not differ in single cultures and showed no mutual influence in co-cultures. In co-cultures with a competitor bacterium belonging to the *Cytophaga–Flavobacterium* group, growth of both *A. hydrophila* strains was reduced while growth of the competitor bacterium was not affected. Exogenous AHL-addition did not influence growth of the *Aeromonas* strains in any microcosm experiment. These results showed that AHL-mediated QS was not required for *A. hydrophila* during colonization and degradation of organic particles in lake water microcosms, suggesting that cell–cell signalling of heterotrophic bacteria in oligotrophic waters relies on novel signal molecules.

Keywords *Aeromonas* · Biofilm · *N*-Acylhomoserine lactones · Oligotrophic lakes · Quorum sensing

Abbreviations

AHL	<i>N</i> -Acylhomoserine lactone
CFU	Colony forming unit
DOM	Dissolved organic matter
QS	Quorum sensing
TCC	Total cell count

Introduction

Heterotrophic bacteria contribute significantly to the degradation of organic detritus particles such as aggregates of decaying phyto- or zooplankton (Azam et al. 1993; Simon et al. 2002; Pernthaler and Amann 2005). After initial attachment, bacteria form biofilms to establish a stable association with organic particles. For the degradation of polymers within organic particles, bacteria release extracellular hydrolytic enzymes. In the course of polymer degradation, the respective oligo- and monomers partly diffuse into the surrounding water and create plumes of dissolved organic matter (DOM) serving as nutrients for suspended heterotrophic bacteria (Azam and Long 2001; Kjørboe and Jackson 2001). In oligotrophic waters like Lake Constance where the concentration of DOM is low [$<1 \text{ mg C l}^{-1}$ (Hanisch et al. 1996)], organic particles represent patches of increased metabolic activity of heterotrophic bacteria (Simon et al. 2002).

It has been demonstrated that various cell–cell interactions of heterotrophic bacteria occur at organic particles (Simon et al. 2002; Grossart et al. 2003). Those can be cooperative interactions, such as biofilm formation or sharing extracellular enzymes and their degradation products,

Communicated by Jörg Overmann.

K. Styp von Rekowski · M. Hempel · B. Philipp (✉)
Fachbereich Biologie, Mikrobielle Ökologie,
Universität Konstanz, Fach M654,
78457 Konstanz, Germany
e-mail: bodo.philipp@uni-konstanz.de

as well as antagonistic interactions, such as production of inhibitory substances (Long et al. 2005). The release of enzymes and secondary metabolites in a highly diffusive environment will be only rewarding at high cell densities. Therefore, these processes are often controlled by cell-density-dependent regulation of gene expression which is mediated by diffusible signal molecules whose concentration correlates with the population density. This process is termed quorum sensing (QS) (for recent reviews see Waters and Bassler 2005; Keller and Surette 2006). In many aquatic Gram-negative bacteria, QS is mediated by *N*-acyl-homoserine lactones (AHLs). AHL-mediated QS is being intensively studied with host-associated bacteria in the context of pathogenicity and symbiosis while possible functions of this process for free-living bacteria in their natural environments have received less attention (Manefield and Turner 2002; Chhabra et al. 2005; Keller and Surette 2006).

The aforementioned bacterial interactions required for detritus utilization render organic particles a paradigmatic setting for QS. Therefore, we hypothesize that AHL-mediated QS has an important function for heterotrophic bacteria from the water column for growth with organic particles. Several observations support this hypothesis. AHL-producing bacteria have been isolated from marine snow (Gram et al. 2002) and AHLs have been detected in aquatic biofilms (McLean et al. 1997). Including AHLs into media for enrichment cultures enhanced the cultivation efficiency for marine (Bruns et al. 2002) and freshwater bacteria (Bussmann et al. 2001). Interestingly, AHL-producing biofilms attract zoospores of green algae (Joint et al. 2002; Tait et al. 2005). While these studies suggest an important function for AHL-mediated QS in aquatic biofilms, a systematic study to prove this function for aquatic heterotrophic bacteria under natural conditions is, to our knowledge, still lacking. To investigate our hypothesis with bacteria from the water column of the littoral zone of Lake Constance we set up enrichment cultures with lake water samples exposed to organic particles in microcosm experiments. If our hypothesis is correct we expect that AHL-producing bacteria enrich at and in vicinity to organic particles and AHLs can be detected. As methodical prerequisites for this study, we had to develop an appropriate microcosm model system for simulating the colonization of organic particles in the littoral zone and to establish a method for a broad screening for AHL-producing bacteria.

Materials and methods

Cultivation of bacteria

Aeromonas hydrophila strains AH-1N and AH-1N *ahyl* (Lynch et al. 2002), *Cytophaga* sp. strain 4D9 (gene bank

accession number BF395377), and all bacterial colonies from enrichments (see below) were cultivated with a HEPES-buffered complex medium designated medium B. Medium B contained the following ingredients (final concentrations): tryptone (0.05%), yeast extract (0.0005%), HEPES (10 mM), NH_4Cl (5 mM), MgSO_4 (0.5 mM), KCl (14 mM), and NaCl (7.2 mM). The pH was adjusted to 7.0 with NaOH. After autoclaving the medium was complemented with CaCl_2 (0.01 mM), phosphate solution (0.15 mM, pH 7, prepared with K_2HPO_4 (0.105 mM) and NaH_2PO_4 (0.045 mM), and trace element solution SL 10 (Widdel et al. 1983). *Chromobacterium violaceum* strain CV026 (McClean et al. 1997) and the *Escherichia coli* strains pSB401, pSB536, and pSB1075 (Winson et al. 1998) were cultivated on Luria-Bertani (LB) medium containing tryptone (1%), yeast extract (0.5%), and NaCl (1%). Solid media were prepared with 1.5% Bacto agar (Difco).

Lake water microcosms for enrichment of heterotrophic bacteria

Lake water microcosms were set up with water samples from the littoral zone of Lake Constance and agarose beads containing autoclaved cells of the unicellular green algae *Scenedesmus obliquus* as nutrient source. The employment of agarose beads was adapted from a study in which they were used to monitor surface attachment of aquatic bacteria (Grossart et al. 2003). Lake water samples were taken with sterile plastic tubes from a pier 10 m offshore the Limnology Institute (University of Constance) at a depth of about 40 cm and directly used for enrichments. For preparation of the agarose beads, 50 ml of *S. obliquus* cultures (kindly provided by E. von Elert) with a total carbon content of 12.6 mg were harvested by centrifugation at $10,000 \times g$ for 10 min at room temperature, washed with 0.9% NaCl, and finally resuspended in 4 ml 0.9% NaCl and 16 ml of distilled water. These 20-ml suspensions were supplied with 1% agarose (Seakem LE agarose, BMA), autoclaved for 20 min at 121°C, and poured into Petri dishes (8.5 cm diameter). From solidified algae-containing agarose, beads with a total algal carbon content of about 0.06 mg were punched out with a truncated 1 ml pipette tip and placed into 7.5 ml lake water in a sterile glass tube. These tubes were incubated at 16°C on a rotatory mixer (CVM, Fröbel) at 100 rpm. After 0, 24, 48, and 72 h samples of the lake water (suspended fraction) and whole agarose beads (biofilm fraction) were taken to determine total cell numbers in the suspended fraction and viable cell numbers in both fractions. To determine the total cell counts (TCCs), cells from the suspended fraction were collected on a black polycarbonate filter (pore size 0.2 µm; Millipore) and stained with 1 µg ml⁻¹ DAPI (4', 6-diamidino-2-phenylindole). Cells on filters were counted with epifluorescence microscopy. To

obtain the biofilm fraction, single agarose beads were repeatedly washed in 0.4 ml medium B. Washed beads were gently homogenized with a sterile plastic pestle in 100 μ l of potassium phosphate buffer (50 mM, pH 6). Homogenates of single beads were further disintegrated enzymatically with 50 units of *Pseudomonas atlantica* agarase (Sigma) at 30°C for 90 min. Viable cells in suspended, washing, and biofilm fractions were quantified by counting colony forming units (CFUs) on medium B agar-plates as described earlier (Klebensberger et al. 2006). Colonies were counted after 24 h incubation at 16°C. TCCs of the biofilm fraction could not be determined because it contained many cell clusters and autofluorescing fragments of algae that interfered with DAPI analysis. Microcosm enrichments were set up on June 25, 2004 (water temperature 14.5°C), and on November 29, 2004 (8°C). As controls, microcosms containing beads without algae were employed.

Lake water microcosms for growth experiments with pure strains in single and co-cultures

Lake water samples were taken as described above and sterilized by filtration through a 0.2 μ m pore membrane (FP30/0.2 CA-S; Schleicher and Schuell). Algae-containing agarose beads were prepared as described above except that the algal carbon content was increased to 0.12 mg. Pre-cultures of *A. hydrophila* strains AH-1N and AH-1N *ahyI* and of *Cytophaga* sp. strain 4D9 were grown with medium B overnight. TCCs of the pre-cultures were determined as described above. Microcosms containing one agarose bead in 5 ml sterile lake water were inoculated with 10⁶ cells ml⁻¹ as single or as co-cultures and incubated as described above. After 24, 48, and 72 h samples from suspended and biofilm fractions were taken and processed as described above. For CFU determination cells were plated on solid medium B with increased tryptone concentration (1%). After overnight incubation, strain 4D9 and the *Aeromonas* strains could be unambiguously differentiated by their different colony morphologies since *Aeromonas* colonies were larger and whitish while *Cytophaga* colonies were smaller and yellow. The *Aeromonas* strains AH-1N and AH-1N *ahyI* were distinguished by the *C. violaceum* CV026 AHL-bioassay (see below).

Bioassays for AHL-production

Single colonies obtained from high dilution steps of the CFU counts of the microcosm experiments were transferred to medium B backup plates for systematic storage. All colonies grown on these backup plates were tested for AHL production in bioassays with *C. violaceum* strain CV026 (McClellan et al. 1997) and the bioluminescent *E. coli* strains pSB401, pSB536, and pSB1075 (Winson et al.

1998; Yates et al. 2002). Bioassays with strain CV026 indicating AHLs with short acyl side chains such as *N*-hexanoyl-L-homoserine lactone (HHL) or *N*-octanoyl-L-homoserine lactone (OHL) were performed by streaking test colonies adjacent to the indicator strain on solid LB medium. AHL-producing bacteria were indicated by violacein production in strain CV026 as described earlier (McClellan et al. 1997). The *E. coli* indicator strains respond to different AHLs by producing bioluminescence; strain pSB401 detects AHLs with short and long acyl side chains, strain pSB536 is specific for butanoyl-L-homoserine lactone (BHL), and strain pSB1075 is specific for AHLs with long acyl side chains such as *N*-3-oxododecanoyl-L-homoserine lactone (OdDHL). For bioassays with the *E. coli* AHL indicator strains, liquid cultures of test colonies were set up with 200 μ l medium B in 96-well microtiter plates (Microwell, Nunc). After incubation for 48 h at 16°C, these microtiter plates were centrifuged at 1,700g for 15 min (5804 R centrifuge with A-2-DWP rotor; Eppendorf). Culture supernatants (50 μ l) from each well were transferred to black 96-well microtiter plates (Microwell optical bottom plates, Nunc) and supplied with 150 μ l of *E. coli* cultures adjusted to an optical density of 0.2 at 600 nm. After 2, 4 and 6 h, bioluminescence and the optical density at 595 nm were measured with a microtiter plate reader (Genios, Tecan). Bioluminescence intensities were normalized to cell densities of the indicator strains. On each black microtiter plate, positive controls were set up with 0.1 μ M (final concentration) of either BHL (Fluka), HHL (Fluka), or OdDHL (kindly provided by Paul Williams) for the respective indicator strains. Equivalent volumes of acetonitrile (the solvent for all AHLs) served as negative controls. Extraction of AHLs from culture supernatants and AHL bioassays after separating extracts by thin layer chromatography (TLC) were performed as described earlier (Yates et al. 2002). As lactone rings in AHLs with short acyl side chains tend to hydrolyze at the slightly alkaline pH values (7.5–8.0) prevailing in the microcosms, we had acidified supernatant samples prior to extraction to reform potentially hydrolyzed lactone rings as described earlier (Yates et al. 2002).

Identification of bacteria

N-acylhomoserine lactone-positive colonies were purified by transferring single colonies on solid medium B several times. Strains still producing AHLs after purification were further characterized by standard phenotypic assays (Gram-typing, oxidase test) and by *MspI* restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene PCR product as described earlier (Schmitt-Wagner et al. 2003). Selected strains with distinct RFLP patterns were further identified by sequencing the 16S rRNA gene.

Sequences were compared with the NCBI-database (<http://www.ncbi.com>) using the BLAST search tool (Altschul et al. 1997).

Results

Enrichment of heterotrophic bacteria in lake water microcosms

For enrichment of particle-associated heterotrophic bacteria from the littoral zone of Lake Constance, the natural microflora was exposed to artificial organic particles consisting of autoclaved cells of the naturally occurring alga *Scenedesmus obliquus* embedded in agarose. In these enrichments, growth of suspended and of particle-associated bacteria (biofilm fraction) was monitored over time. TCCs and CFUs of suspended bacteria increased in all microcosms, and the highest numbers of cells were obtained in microcosms containing agarose beads with algae (Fig. 1a, b). Cultivation efficiencies (CFU/TCC) of suspended bacteria peaked after 48 h incubation in microcosms with algae-containing agarose beads and reached values above 60%. In control microcosms containing agarose beads without algae CFU-counts increased less and cultivation efficiencies were around 1% throughout the experiments. The cultivation efficiency in lake water microcosms without agarose beads was in the same range (not shown). In the biofilm fractions, CFUs in microcosms with algae-containing agarose beads increased much stronger than those from microcosms

containing agarose beads without algae (Fig. 1c). As described under **Materials and methods** TCCs of the biofilm fraction could not be determined for technical reasons. The determination of CFUs in the washing fractions revealed that loosely attached bacteria could be removed (not shown). In summary, algae-containing agarose beads supported growth of heterotrophic bacteria in suspension and in the biofilm fraction. The growth observed in microcosms without algae was also observed in microcosms without any agarose beads (not shown), indicating that this growth must be based on endogenous nutrients from the lake water. The enrichments set up in June (not shown) and November 2004 (Fig. 1) gave similar results.

Screening for and identification of AHL-producing bacteria

All colonies growing on medium B backup plates were screened for AHL-production. As some colonies did not grow on solid LB medium they could not be tested with the indicator strain CV026. This indicator strain responds to AHLs with short acyl side chains only and has a relatively low sensitivity. To overcome these limitations, we developed a screening method of culture supernatants in 96-well microtiter plates using three highly sensitive bioluminescent *E. coli* indicator strains that cover a broad range of AHLs with different acyl side chains (Fig. 2). This procedure allowed a fast screening of a total number of 905 colonies for AHL-production. We found that 244 colonies stimulated indicator strains. Many AHL-producing colonies activated two or more indicator strains, namely strains

Fig. 1 Lake water microcosms for enrichment of heterotrophic bacteria from the littoral zone of Lake Constance. The microcosms contained agarose beads with algae (filled square) and without algae (open square). **a** TCCs of the suspended fraction, **b** CFUs of the suspended and **c** the biofilm fraction. **d** Estimated CFUs of AHL-producing bacteria in the suspended fraction (A, B) and the biofilm fraction (C, D) of microcosms containing agarose beads without (open bars) and with algae (filled bars). Microcosms were set up in November 2004. Bars represent standard deviation ($n = 6$)

Fig. 2 Response of bioluminescent *Escherichia coli* indicator strains to different AHL-concentrations after an incubation time of 4 h: (open square) strain pSB 401 with HHL, (open circle) strain pSB 536 with BHL, (open triangle) strain pSB 1075 with OdDHL. Bars represent standard deviation ($n = 3$)

CV026 and pSB536 or strains pSB401 and pSB1075. There were no clear correlations between the origin of colonies (regarding incubation time of and localization within microcosms) and their activation of indicator strains.

The percentages of AHL-producing colonies from total CFUs were determined and used to estimate the total numbers of AHL-producing CFU (Fig. 1d). In all microcosms, AHL-producing bacteria enriched over time. The highest numbers were observed in microcosms with algae-containing agarose beads. After 48 h, there were more than 10^6 AHL-producing colonies ml^{-1} in the suspended fraction of these microcosms, corresponding to a 10^4 -fold enrichment compared to freshly collected lake water. Considering the TCCs, cultivable AHL-producing bacteria accounted for about 15% of the total bacterial number. To investigate whether the enrichment of AHL-producing bacteria was reflected by the accumulation of AHLs we analyzed supernatants of lake water microcosms with the bioluminescent *E. coli* strains. AHLs could not be detected in any of the microcosm supernatants, not even in 100 μl extracts of 50 ml lake water microcosms including 20 agarose beads with algae [detection limits: 10 nM for *E. coli* pSB 401 and *E. coli* pSB 536, 0.5 nM for *E. coli* pSB1075 (Fig. 2)].

From 244 AHL-producing colonies, 193 AHL-producing pure cultures were isolated and characterized. All AHL-producing isolates were oxidase-positive, Gram-negative bacteria. RFLP-analysis with *MspI* showed very similar patterns for all isolates. Sequencing the 16S rRNA gene from selected isolates revealed that all of them belonged to the genus *Aeromonas*, including *A. hydrophila*, *A. sobria*, *A. salmonicida*, and *A. veronii*. TLC-analysis of supernatants from selected strains grown with medium B indicated production of different AHLs with short acyl side chains,

including BHL, HHL, and OHL (not shown). To investigate whether these *Aeromonas* strains would produce AHLs in lake water microcosms we cultivated selected *Aeromonas* strains in filter-sterilized lake water containing agarose beads with algae. No AHLs could be detected in organic extracts of small (7.5 ml) or large (200 ml) lake water microcosms.

Growth experiments in lake water microcosms

To test investigate whether AHL-mediated QS had an important function for growth of *Aeromonas* sp. with organic aggregates, we submitted *A. hydrophila* strain AH-1N and the AHL-deficient isogenic mutant strain AH-1N *ahyI* (Lynch et al. 2002) to growth experiments in lake water microcosms. With these two strains it has been shown previously that AHL-based QS is important for biofilm formation (Lynch et al. 2002) and exoenzyme production (Swift et al. 1999). Thus, these strains were feasible representatives of *Aeromonas* sp. to test for the function of AHL-mediated QS in our lake water microcosms. In growth experiments with pure *Aeromonas* cultures, the wild-type strain AH-1N and the *ahyI* mutant did not differ regarding growth rates and cell numbers. Both strains reached 10^7 CFUs ml^{-1} in the suspended fraction after 48 h and about 10^7 CFUs ml^{-1} after 72 h in the biofilm fraction. To investigate whether the wild type had a growth advantage in competition with the *ahyI* mutant the strains were inoculated at a 1:1 ratio. This ratio remained stable in the suspended and in the biofilm fraction throughout the experiment, indicating that both strains grew equally (Fig. 3a, b). To investigate whether the *ahyI* mutant might profit from AHL-production by the wild type, the strains were inoculated at a 9:1 ratio of wild type to *ahyI* mutant. This initial ratio remained also stable throughout growth (not shown). These results indicated that the ability to produce AHLs did not affect growth of *A. hydrophila* strain AH-1N in lake water microcosms.

To investigate whether cell-cell communication via AHLs might be important for *A. hydrophila* in competition with other bacteria, we co-inoculated the *Aeromonas* strains with *Cytophaga* sp. strain 4D9, which we had isolated from a lake water microcosm. In general, members of the *Cytophaga-Flavobacterium* group of the *Bacteroidetes* are abundant in aquatic habitats and are important degraders of polymers (Kirchman 2002; Lemarchand et al. 2006). We isolated several *Cytophaga-Flavobacterium* strains from high dilution steps of our microcosms. These traits rendered a *Cytophaga* strain a feasible competitor for *A. hydrophila* in ecophysiological experiments. In pure culture, strain 4D9 grew in the suspended and in the biofilm fraction, reaching higher CFUs than the *Aeromonas* strains (not shown). If co-inoculated with strains AH-1N or AH-1N *ahyI* individually, strain 4D9 grew like the pure culture

Fig. 3 Growth of pure strains in defined co-cultures in lake water microcosms. CFUs of a co-culture of *Aeromonas hydrophila* strains AH-1N (filled square) and *ahyI* (open square) in (a) suspended and (b) biofilm fractions. CFUs of co-cultures of *Cytophaga* strain 4D9 (filled circle) with strain AH-1N (filled square) and of strain 4D9 (open circle) with strain *ahyI* (open square) in (c) suspended and (d) biofilm fractions. Bars represent standard deviation ($n = 9$)

(Fig. 3c, d). The same result was obtained if strain 4D9 was co-inoculated with a 1:1 mixture of strains AH-1N and AH-1N *ahyI* (not shown). These results indicated that growth of *Cytophaga* strain 4D9 was not influenced by the *Aeromonas* strains. In contrast, growth of the *Aeromonas* strains was severely influenced by strain 4D9. Both strains showed only poor growth in co-cultures with strain 4D9 (Fig. 3c, d). In suspended fractions, CFUs of both *Aeromonas* strains first decreased after 24 h and then increased to the initial values which remained stable for the remaining incubation time (Fig. 3c). In the biofilm fractions, CFU counts of both *Aeromonas* strains decreased with time by one order of magnitude (Fig. 3d). No difference between the wild type and the *ahyI* mutant could be observed.

We could not detect AHLs in any microcosm containing strain AH-1N. To investigate whether the addition of AHLs would influence growth of the *Aeromonas* strains, we supplied lake water microcosms with BHL (1 μ M), the cognate AHL of *A. hydrophila* strain AH-1N. This addition had no effect on growth of strains AH-1N, AH-1N *ahyI*, and 4D9, neither in pure nor in mixed cultures (not shown). Monitoring BHL in supernatants with *E. coli* pSB536 revealed that the concentration of BHL decreased with time in all lake water microcosms including the sterile control, presumably due to chemical hydrolysis of the lactone ring.

Discussion

Quorum sensing via AHLs has been shown to be involved in biofilm formation (Kjelleberg and Molin 2002) and

competitive or cooperative bacterial interactions (Keller and Surette 2006) in many Gram-negative bacteria. In this study, we scrutinized whether AHL-producing bacteria from oligotrophic lake water would enrich at or in vicinity of organic particles and, if so, whether a potential function of AHL-signaling could be demonstrated in ecophysiological experiments with pure strains in single or defined co-cultures. As a prerequisite for these investigations we had to establish lake water microcosms with artificial organic particles which should provide conditions that are very similar to the natural situation in the littoral and that should allow retrieval of particle-associated biofilm bacteria in a reproducible way. The agarose beads containing *Scenedesmus obliquus* cells met these requirements. While the algal cells served as a nutrient source with a defined carbon concentration for growth of heterotrophic bacteria, the agarose beads themselves were not degraded during the incubation period and served as a mere matrix for algal detritus. The number of bacteria in the suspended fraction of algae-containing microcosms did not exceed cell numbers usually prevailing during algal blooms in Lake Constance (Güde et al. 1985), indicating that the carbon concentration of *S. obliquus* was set in a realistic range. Furthermore, the agarose beads could be disintegrated under comparably mild and defined conditions, allowing qualitative retrieval of particle-associated biofilm bacteria. A second important prerequisite for our study was the establishment of a method that allowed fast screening of a large number of bacteria for AHL-production. The bioassays with highly sensitive *Escherichia coli* indicator strains in 96-well format proved to be a reliable and efficient screening that

covered AHLs of different structures and allowed analysis of bacteria that did not grow on solid LB medium.

We found that *Aeromonas* sp. with the ability to produce AHLs enriched in the biofilm and in the suspended fraction of lake water microcosms with algae-containing agarose beads. These *Gammaproteobacteria* are ubiquitous in aquatic environments from which they can be readily isolated (von Graevenitz 1987). They occur at low numbers of $10\text{--}10^3$ CFU ml⁻¹ in surface waters and show comparably low survival rates in oligotrophic waters (Hazen et al. 1978; Kersters et al. 1996). Although fast-growing *Gammaproteobacteria* are usually not abundant in natural aquatic systems it has been repeatedly observed that they can transiently enrich at patches of high nutrient availability (Pernthaler and Amann 2005). In agreement with this observation, we showed that cell numbers of *Aeromonas* strains increased to 10^6 CFU ml⁻¹ at nutrient hot spots in oligotrophic lake water microcosms. As some *Aeromonas* species are pathogenic for fish (Bruhn et al. 2005) and for humans (Leclerc et al. 2002; von Graevenitz 2007) our study calls attention to detritus particles as potential reservoirs of these opportunistic pathogens.

At first sight, the enrichment of *Aeromonas* strains with the ability to form AHLs suggested an important role of AHL-mediated QS for these strains during colonization and degradation of organic particles, thereby supporting our initial hypothesis. However, we could not detect AHLs in any microcosm, not even in 500-fold concentrated extracts, indicating that the natural microflora did not produce significant amounts of AHLs. Nevertheless, AHL-signaling could operate in microcompartments on the organic particles. Diffusion sensing (Redfield 2002) and compartment sensing (Koerber et al. 2005) are modified concepts of QS in which cell–cell communication could occur at low cell numbers if these cells reside in a habitat of low diffusion or if they are clustered in aggregates. A feasible approach to test these alternative concepts of AHL-mediated QS in the lake water microcosms was to compare pure cultures of an AHL-producing *Aeromonas* strain with an isogenic AHL-defective mutant. AHL-mediated diffusion sensing or compartment sensing should be indicated by reduced growth of the AHL-deficient mutant strain *ahyI* in the biofilm fraction. However, we did not obtain any evidence of such a growth disadvantage of the *ahyI* mutant, neither in pure culture nor in competition with the wild type or with *Cytophaga* sp. strain 4D9. Alternatively, the *ahyI* mutant could have a growth advantage during co-incubation with the wild type because it could profit from the AHL-signal while avoiding energy investment for its synthesis. A selective advantage of such so-called cheaters has been postulated for QS-deficient mutants (Kreft 2005; Keller and Surette 2006). However, the *ahyI* mutant had no growth advantage under conditions prone to cheating. As the *ahyI*

mutant had no phenotype in any microcosm experiment and as the addition of BHL to microcosm experiments had no effects, we conclude that AHL-mediated QS is not required for growth of *A. hydrophila* with organic particles in microcosms simulating the conditions in the littoral of Lake Constance. It must be emphasized that AHL-based cell–cell communication below the detection limit of our indicator strains might occur in microcolonies but it cannot be a prerequisite for growth in this environment. To our knowledge, this is the first systematic attempt to explore the role of AHL-mediated QS for degradation of detritus particles under oligotrophic conditions.

As pointed out in the introduction, the utilization of organic particles by heterotrophic bacteria in aquatic systems is highly prone to involve QS. Why did *A. hydrophila* not employ AHL-mediated QS although it is undoubtedly required for the production of extracellular enzymes (Swift et al. 1999; Bi et al. 2007) and for biofilm formation (Lynch et al. 2002)? In the respective experiments, *A. hydrophila* was cultivated in rich media. As AHLs are widespread signal molecules among bacteria of different species their usage implies risks for the producers, such as the aforementioned cheating, unwanted activation of competitors, or signal inactivation by AHL-degrading bacteria (Kreft 2005). Under the nutrient- and energy-limited conditions prevailing in our microcosm experiments, energy investment into QS via such unspecific signals may not pay off. AHL-based signaling may be more important in nutrient-rich environments. In agreement with this assumption AHLs could be extracted from infected fish (Bruhn et al. 2005) and the addition of AHLs to activated sludge had an influence on the bacterial community composition (Valle et al. 2004).

In conclusion, our study reveals that AHLs are not universal signal molecules that bacteria would employ under all environmental conditions, thereby endorsing the search for novel bacterial signal molecules.

Acknowledgments We acknowledge Miguel Cámara and Paul Williams (Nottingham) for gift of the AHL indicator strains, the *Aeromonas hydrophila* strains, and of OdDHL. We thank Elisabeth Gross for discussions and Bernhard Schink for continuous support. This work was funded by the German Science Foundation (DFG) in the Collaborative Research Center SFB454, project B9, to B.P..

References

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402
- Azam F, Long RA (2001) Sea snow microcosms. *Nature* 414:495, 497–498
- Azam F, Smith DC, Steward GF, Hagström Å (1993) Bacteria-organic matter coupling and its significance for oceanic carbon cycling. *Microb Ecol* 28:167–179

- Bi ZX, Liu YJ, Lu CP (2007) Contribution of AhyR to virulence of *Aeromonas hydrophila* J-1. Res Vet Sci. doi:10.1016/j.rvsc.2007.01.003
- Bruhn JB, Dalsgaard I, Nielsen KF, Buchholtz C, Larsen JL, Gram L (2005) Quorum sensing signal molecules (acylated homoserine lactones) in gram-negative fish pathogenic bacteria. Dis Aquat Organ 65:43–52
- Bruns A, Cypionka H, Overmann J (2002) Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central Baltic Sea. Appl Environ Microbiol 68:3978–3987
- Bussmann I, Philipp B, Schink B (2001) Factors influencing the cultivability of lake water bacteria. J Microbiol Methods 47:41–50
- Chhabra SR, Philipp B, Eberl L, Givskov M, Williams P, Cámara M (2005) Extracellular communication in bacteria. In: Schulz S (eds) Topics in current chemistry. Springer GmbH, pp 279–315
- Gram L, Grossart HP, Schlingloff A, Kjørboe T (2002) Possible quorum sensing in marine snow bacteria: production of acylated homoserine lactones by *Roseobacter* strains isolated from marine snow. Appl Environ Microbiol 68:4111–4116
- Grossart HP, Kjørboe T, Tang K, Ploug H (2003) Bacterial colonization of particles: growth and interactions. Appl Environ Microbiol 69:3500–3509
- Güde H, Haibel B, Müller H (1985) Development of planktonic bacterial populations in a water column of Lake Constance (Bodensee–Obersee). Arch Hydrobiol 105:59–77
- Hanisch K, Schweitzer B, Simon M (1996) Use of dissolved carbohydrates by planktonic bacteria in a mesotrophic lake. Microb Ecol 31:41–55
- Hazen TC, Fliermans CB, Hirsch RP, Esch GW (1978) Prevalence and distribution of *Aeromonas hydrophila* in the United States. Appl Environ Microbiol 36:731–738
- Joint I, Tait K, Callow ME, Callow JA, Milton D, Williams P, Cámara M (2002) Cell-to-cell communication across the prokaryote-eukaryote boundary. Science 298:1207
- Keller L, Surette MG (2006) Communication in bacteria: an ecological and evolutionary perspective. Nat Rev Microbiol 4:249–258
- Kersters I, Huys G, Van Duffel H, Vancanneyt M, Kersters K, Verstrate W (1996) Survival potential of *Aeromonas hydrophila* in freshwaters and nutrient-poor waters in comparison with other bacteria. J Appl Bacteriol 80:266–276
- Kjørboe T, Jackson GA (2001) Marine snow, organic solute plumes, and optimal chemosensory behavior of bacteria. Limnol Oceanogr 46:1309–1318
- Kirchman DL (2002) The ecology of *Cytophaga-Flavobacteria* in aquatic environments. FEMS Microbiol Ecol 39:91–100
- Kjelleberg S, Molin S (2002) Is there a role for quorum sensing signals in bacterial biofilms? Curr Opin Microbiol 5:254–258
- Klebensberger J, Rui O, Fritz E, Schink B, Philipp B (2006) Cell aggregation of *Pseudomonas aeruginosa* strain PAO1 as an energy-dependent stress response during growth with sodium dodecyl sulfate. Arch Microbiol 185:417–427
- Koerber AJ, King JR, Williams P (2005) Deterministic and stochastic modelling of endosome escape by *Staphylococcus aureus*: “quorum” sensing by a single bacterium. J Math Biol 50:440–488
- Kreft J-U (2005) Conflicts of interest in biofilms. Biofilms 1:265–276
- Leclerc H, Schwartzbrod L, Dei-Cas E (2002) Microbial agents associated with waterborne diseases. Crit Rev Microbiol 28:371–409
- Lemarchand C, Jardillier L, Carrias JF, Richardot M, Debroas D, Sime-Ngando T, Amblard C (2006) Community composition and activity of prokaryotes associated to detrital particles in two contrasting lake ecosystems. FEMS Microbiol Ecol 57:442–451
- Long RA, Rowley DC, Zamora E, Liu J, Bartlett DH, Azam F (2005) Antagonistic interactions among marine bacteria impede the proliferation of *Vibrio cholerae*. Appl Environ Microbiol 71:8531–8536
- Lynch MJ, Swift S, Kirke DF, Keevil CW, Dodd CE, Williams P (2002) The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. Environ Microbiol 4:18–28
- Manefield M, Turner SL (2002) Quorum sensing in context: out of molecular biology and into microbial ecology. Microbiology 148:3762–3764
- McClellan KH, Winson MK, Fish L, Taylor A, Chhabra SR, Cámara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GS, Williams P (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiology 143:3703–3711
- McLean RJ, Whiteley M, Stickler DJ, Fuqua WC (1997) Evidence of autoinducer activity in naturally occurring biofilms. FEMS Microbiol Lett 154:259–263
- Pernthaler J, Amann R (2005) Fate of heterotrophic microbes in pelagic habitats: focus on populations. Microbiol Mol Biol Rev 69:440–461
- Redfield RJ (2002) Is quorum sensing a side effect of diffusion sensing? Trends Microbiol 10:365–370
- Schmitt-Wagner D, Friedrich MW, Wagner B, Brune A (2003) Phylogenetic diversity, abundance, and axial distribution of bacteria in the intestinal tract of two soil-feeding termites (*Cubitermes* spp.). Appl Environ Microbiol 69:6007–6017
- Simon M, Grossart HP, Schweitzer B, Ploug H (2002) Microbial ecology of organic aggregates in aquatic ecosystems. Aquat Microb Ecol 28:175–211
- Swift S, Lynch MJ, Fish L, Kirke DF, Tomas JM, Stewart GS, Williams P (1999) Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infect Immun 67:5192–5199
- Tait K, Joint I, Daykin M, Milton DL, Williams P, Cámara M (2005) Disruption of quorum sensing in seawater abolishes attraction of zoospores of the green alga *Ulva* to bacterial biofilms. Environ Microbiol 7:229–240
- Valle A, Bailey MJ, Whiteley AS, Manefield M (2004) *N*-acyl-L-homoserine lactones (AHLs) affect microbial community composition and function in activated sludge. Environ Microbiol 6:424–433
- von Graevenitz A (1987) Research on *Aeromonas* and *Plesiomonas*. Experientia 43:348–358
- von Graevenitz A (2007) The role of *Aeromonas* in diarrhea: a review. Infection 35:59–64
- Waters CM, Bassler BL (2005) Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 21:319–346
- Widdel F, Kohring G-W, Mayer F (1983) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. Arch Microbiol 134:286–294
- Winson MK, Swift S, Fish L, Throup JP, Jørgensen F, Chhabra SR, Bycroft BW, Williams P, Stewart GS (1998) Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. FEMS Microbiol Lett 163:185–192
- Yates EA, Philipp B, Buckley C, Atkinson S, Chhabra SR, Sockett RE, Goldner M, Dessaux Y, Cámara M, Smith H, Williams P (2002) *N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect Immun 70:5635–5646