Quantitative role of shrimp fecal bacteria in organic matter fluxes in a recirculating shrimp aquaculture system

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Abstract

Microorganisms play integral roles in the cycling of carbon (C) and nitrogen (N) in recirculating aquaculture systems (RAS) for fish and shellfish production. We quantified the pathways of shrimp fecal bacterial activities and their role in C- and N-flux partitioning relevant to culturing Pacific white shrimp, *Penaeus (Litopenaeus) vannamei*, in RAS. Freshly produced feces from *P. vannamei* contained 0.6–7 × 10¹⁰ bacteria g⁻¹ dry wt belonging to *Bacteroidetes* (7%), *Alphaproteobacteria* (4%), and, within the *Gammaproteobacteria*, almost exclusively to the genus *Vibrio* (61%). Because of partial disintegration of the feces (up to 27% within 12 h), the experimental seawater became inoculated with fecal bacteria. Bacteria grew rapidly in the feces and in the seawater, and exhibited high levels of aminopeptidase, chitinase, chitobiase, alkaline phosphatase, α- and β-glucosidase, and lipase activities. Moreover, fecal bacteria enriched the protein content of the feces within 12 h, potentially enriching the feces for the coprophagous shrimp. The bacterial turnover time was much faster in feces (1–10 h) than in mature RAS water (350 h). Thus, shrimp fecal bacteria not only inoculate RAS water but also contribute to bacterial abundance and productivity, and regulate system processes important for shrimp health.

Introduction

Microorganisms play important roles in aquaculture systems with regard to disease, water quality, and as potential food for the target species (Moss, 2002; Balcázar et al., 2006). However, a process-oriented ecosystem approach to studying microbial community structure and function is lacking in these systems. In marine habitats, microorganisms strongly influence carbon- (C), nitrogen- (N), and phosphorus-flux pathways via metabolic interactions with dissolved (DOM) and particulate organic matter (POM) (Pomeroy et al., 2007). The principles underlying bacteria–organic matter interactions in the marine systems are also applicable to aquaculture. Thus, Moriarty (1986) showed that bacterial production in shrimp ponds increased with increasing additions of feed and concluded that most of the feed was, in fact, used by bacteria to cover their C demand. Moriarty (1997) combined microbial abundance and production measurements with the flow of organic matter through the food web to develop the first integrative model of C- and N-flux partitioning in a marine aquaculture system. This approach has advanced system N budgeting, i.e., quantifying feed N utilization vs. N exports into adjacent waters, sediments, and the atmosphere (Funge-Smith & Briggs, 1998; Burford & Williams, 2001; Ebeling et al., 2006).

Shrimp farming is traditionally carried out in coastal earthen ponds where flow-through water maintains acceptable water quality (Hopkins et al., 1993). However, the influent water can be a vector for viral and bacterial pathogens (Lotz, 1997), and the effluent adversely affects coastal water quality (Naylor & Burke, 2005). Recirculating aquaculture systems (RAS) offer an attractive alternative (Sandifer & Hopkins, 1996; Otoshi et al., 2009), considerably reducing water requirements and thereby minimizing pathogen introduction into RAS and pollution of the environment (Moss, 2002).

Microbial communities are an integral part of RAS for fish and shellfish production (Moss, 2002; Cytryn et al., 2005). Yet, in shrimp RAS, there is a paucity of information on microbial community structure, microbially mediated processes, and the complex relationships among microorganisms, system inputs, water quality, and shrimp health. Shrimp RAS can exhibit rapid changes in ambient nutrient concentrations, which result in unpredictable blooms and crashes of eukaryotic microalgae (Otoshi et al., 2006). Nutrient fluxes result from periodic
exogenous organic matter inputs and poorly understood changes in microbial cycling. In addition, shifts in heterotrophic bacterial community structure can favor opportunistic pathogens endangering shrimp health. A controlled ecosystem approach to RAS could eventually lead to a mechanistic understanding of the regulation of microbial processes and their interactions with the environment and the species under culture. This could enhance understanding of system function, allow predictions about system stability, and provide better tools for system management.

We evaluated the fate of shrimp feed and feces to better understand feed-derived C and N turnover pathways and flux partitioning between different biological compartments in shrimp RAS. Feed and shrimp feces represent significant sources of organic matter in these RAS and hence influence nutrient flow pathways. Although shrimp feces are densely populated with bacteria (Harris, 1993 and references therein), their contribution to organic matter turnover in RAS remains unknown. In small-scale experiments, we determined the abundance, composition, productivity, and hydrolytic enzyme activities of bacteria in shrimp feces and compared them with those of free-living bacteria in a RAS used for super-intensive shrimp production. We then considered the significance of bacteria-mediated processes for shrimp health and aquaculture system stability.

Materials and methods

Experimental design

In order to quantify the fate of shrimp feed and feces and assess the role of bacteria in feed C- and N-flux pathways, we conducted a series of experiments. First, gut passage time and feed ingestion and egestion rates were determined in single shrimp assays to study feed processing by the shrimp. Second, the fate of not immediately consumed shrimp feed was studied by exposing the feed to sterile seawater and analyzing the release of C and N into the surrounding water. These first two studies were conducted to provide background information and context for the primary objective of this study, where we determined the fate of shrimp feces and the role of shrimp gut bacteria in C and N fluxes. This was accomplished by incubating shrimp feces for 12 h in sterile seawater and quantifying shrimp gut-derived microbial community structure, turnover rates, and enzyme activities. These data were compared with similar information from RAS water where the shrimp were raised.

Characterization and maintenance of experimental shrimp

Pacific white shrimp, Penaeus (Litopenaeus) vannamei (~18 g live body weight), from a RAS at Oceanic Institute (Waimanalo, HI) were acclimated in 50 L, aerated, flow-through aquaria for several days before the experiments, and fed ad libitum with a commercial feed (based on fishmeal, 35% protein, 2% squid; Zeigler Bros. Inc., Gardners, PA). Seawater in the aquaria was from a seawater aquifer (temperature 29±1 °C; salinity 34–35 psu).

Feed processing by shrimp

To determine gut passage time, feed pellets were stained with Coomassie Brilliant Blue (Sigma; 2% w/v in 0.2 μm-filtered, deionized water) and dried at 65 °C. Preliminary experiments showed that shrimp did not discriminate between stained and unstained feed. Six shrimp were placed individually in clear plastic containers with 3–5 L seawater aerated with an air stone. After 1 h, small amounts of unstained feed were offered; hence, shrimp did not starve and potentially alter their feeding behavior. After 1 h, feces and excess feed were siphoned out of the containers. The guts of all shrimp were visibly full (i.e. all shrimp were actively feeding). Stained feed was then added and feeding activity and shrimp behavior were observed. Gut passage time was calculated as the interval between the initial ingestion of stained feed and the first observation of blue feces.

Ingestion and egestion rates were quantified in a second, single-shrimp assay (as above). Six new P. vannamei were fed measured amounts of unstained feed until sated (i.e. feed ingestion stopped) so that feed availability was not limited and the amount of feed that had to be retrieved from the containers was minimized. After 4 h, uneaten feed and feces, distinguishable by their appearance, were separately siphoned and weighed. The water content of the feed and feces was determined by drying at 60 °C.

Feed stability in seawater

Dry, autoclaved feed was analyzed for total organic carbon (TOC), total organic nitrogen (TON), and protein content. For evaluating the feed stability, feed was incubated in autoclaved, 0.2 μm-filtered seawater and the increase in dissolved organic carbon (DOC), dissolved nitrogen (DN), and protein in the seawater was measured over time (0, 4, 8, and 12 h). At t12h, the feed was analyzed for TOC, TON, and protein (see methods below).

Feces incubation experiments

Experiment A: Twenty shrimp from aquaria were starved for 16 h, rinsed with autoclaved seawater, and transferred to four 50 L aquaria (five shrimp per aquarium) containing 0.2 μm-filtered autoclaved seawater. Feces produced during the first 2 h were discarded to avoid contamination with environmental bacteria. Shrimp were refed with autoclaved
feed, fresh feces were collected immediately, and stored at 4 °C until ~4 g of wet feces had been collected (<4 h).

Triplicate feces samples were weighed for initial (t₀) biochemical and microbial measurements. Additional triplicate feces samples were weighed (300–350 mg wet feces per sample) and incubated in glass beakers containing 1 L autoclaved, 0.2 µm-filtered seawater (30 °C; 12 h; in the dark). The beakers were periodically swirled gently. Seawater was sampled after 4, 8, and 12 h and at 12 h the feces were collected in weighing dishes and blotted with paper tissue before weighing. Feces and seawater subsamples were analyzed for TOC, TON, DOC, protein, water content, bacterial abundance, biomass, community composition, production, and hydrolytic ectoenzyme activity. Bacterial production and hydrolase activities were measured immediately following incubation. Subsamples for activity parameters at t₀ were acclimated to 30 °C for 10 min before analysis.

The above experiment was repeated twice (Experiments B and C) with a reduced set of parameters. In Experiment B, we evaluated the effects of feces storage at 4 °C on bacterial activity; in Experiment C, we focused on the relationship between changes in bacterial abundances and fecal protein. Shrimp were sacrificed, and their guts were removed aseptically and fixed for analysis for bacterial community composition. We also analyzed RAS water, from which the shrimp guts, and water samples (Experiment A) were analyzed using FISH as in Pernthaler et al. (2001). Cells were hybridized at 46 °C with 5'-Cy3-labeled oligonucleotides (ThermoHybaid, Ulm, Germany). Probes were targeted to all bacteria (EUB338, EUB338-II, EUB338-III, Daims et al., 1999), Bacteroidetes (CF319a), Betaproteobacteria (BET42a), Gammaproteobacteria (GAM42a, all Manz et al., 1992), Vibrio spp. (GV822, Giuliano et al., 1999), and Alphaproteobacteria (ALF968, Neef, 1997). The EUB338 antisense probe (NON338, Wallner et al., 1993) served as a negative control. Hybridization conditions were according to the references for the probes; the washing temperature was 48 °C. Hybridized samples were counterstained with DAPI-Vecta Shield, visualized by EFM at × 1000 using green light excitation for FISH signals, and counted as % of all DAPI-stained cells in identical microscopic fields. On average, 700 DAPI-stained cells were counted per filter.

**Bacterial size and biomass**

Bacterial dimensions were measured from digital images of cells hybridized with EUB338 I–III probes using the software AXIOVISION 4 (Zeiss, Jena, Germany). Measurements were made from 50–100 cells per sample. Assuming spherical and cylindrical shapes for cocci- and rod-shaped cells, respectively, the mean cell volumes and total bacterial volumes were calculated. Differences in the mean cell volumes at t₀ and t₁₂h were analyzed using Student’s t-tests. Cell protein content and carbon were computed as in Simon & Azam (1989), assuming protein is 50% of cell dry weight.

**Bacterial production**

Bacterial community productivity in feces and water samples collected during Experiment A at t₀ and t₁₂h and from RAS water were analyzed by immunodetection of incorporated 5-bromo-2'-deoxyuridine (BrdU) (Steward & Azam, 1999; Nelson & Carlson, 2005). Aliquots of water samples and feces suspended in Milli-Q water were spotted onto 0.2-µm Nylon membrane (Osmonics) in a slot blotter and treated as described by Steward & Azam (1999). Immobilized samples and standards were conjugated with BrdU Antibody-HRP Fab (Roche Applied Science) and subsequently with the Super-Signal-West-Femto substrate (Pierce Biotechnology, Rockford, IL) as described by Nelson & Carlson (2005). Chemiluminescence was quantified by scanning the membranes on a flatbed scanner (Typhoon 9410) and signal intensity was determined using IMAGEQUANT.

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(both, GE Healthcare-Amersham, Piscataway, NJ). Signal intensity was converted to BrdU incorporation using a BrdU standard curve prepared from 0.6 μm-filtered seawater incubated for 24 h with either 20 nM BrdU or 20 nM BrdU+1% 3H-BrdU (Moravek Biochemicals, Brea, CA) as in Steward & Azam (1999). A conversion factor of 2 × 10^{18} cells produced per mole BrdU incorporated was used (Fuhrman & Azam, 1982) to calculate cell production.

**Hydrolytic ectoenzyme activities**

Enzyme activities were measured from the rate of hydrolysis of fluorogenic substrate analogues (Hoppe, 1983) using a Hoefer TKO-100 fluorometer (excitation 356 nm, emission 460 nm). The substrates were L-leucine-4-methylcoumarinyl-7-amide (Leu-AMC) for aminopeptidase activity and the 4-methylumbelliferone (MUF)-labeled substrates α-D-glucoside, β-D-glucoside, phosphate, N-acetyl-β-D-glucosaminide, N-acetyl-β-D-N,N'-diacetylcitobioside, and stearate for α- and β-glucosidase, alkaline phosphatase, chitinase, chitobiosidase, and lipase activities, respectively. Substrates were dissolved in 2-methoxyethanol (and added to 40 μM in Experiment A and 100 μM in Experiment B). Triplicate samples from feces (suspended in autoclaved, 0.2 μm-filtered seawater) and seawater from the experiments as well as the RAS were incubated at 30 °C. Heat-killed samples served as controls. Fluorescence was calibrated with the Leu-AMC and MUF standard curves.

**Particulate and dissolved organic carbon and nitrogen**

Feces, feed, seawater, and RAS samples were collected on precombusted glass fiber filters (Whatman GF/F) in triplicate and stored at −20 °C. Analyses of particulate organic carbon (POC) and nitrogen (PON) were performed at the MSI Analytical Lab (University of California Santa Barbara, CA) on a CEC 440HA Elemental Analyzer (Control Equipment Corp., MA). For DOC analysis, GF/F filtrates were acidified to < pH 2 with H₂PO₄ and kept refrigerated until analysis by high-temperature catalytic oxidation (Sugimura & Suzuki, 1988) using a TOC-5000 analyzer (Shimadzu). Standards were made of potassium hydrogen phthalate. For samples from Experiments B and C, simultaneous DOC and DON analyses were performed with a TOC-V TNM-1 analyzer (Shimadzu) using nicotinic acid as the DN standard.

**Protein content**

Feces and feed samples were analyzed for total hydrolyzed amino acids (THAA) to compute the protein content. Samples dispensed in 1 mL Milli-Q water were flame sealed under N₂ and hydrolyzed for 24 h at 100 °C. Extracts were evaporated to dryness in a vacuum centrifuge at 60 °C and exposed to 6 M HCl under a N₂ atmosphere for 24 h at 100 °C (vapor-phase acid hydrolysis). The residues were solubilized in Milli-Q water, desalted (Glavin et al., 2004), and concentrated by vacuum centrifugation. o-Phthalaldehyde (Sigma) was added to aliquots and A_{340nm} was measured photometrically after 90 s. Protein concentration (as THAA) was calculated based on standards prepared according to the amino acid concentrations of the feed (in mM: Gln: 1.0; Glu: 1.7; Asn: 0.8; Asp: 1.5; Gly: 2.7; Leu: 1.5; Ile: 1.5; Phe: 1.2; Val: 1.7; Ala: 1.7; Arg: 1.1; Thr: 1.3; Tyr: 0.3; Ser: 1.4; His: 0.6). Differences in the mean protein concentrations at t₀ and t₁₂h were analyzed using Student's t-tests.

**Results**

**Feed processing by shrimp**

*Penaeus vannamei* broke up the feed pellets (2.2–6.7 mm length, 2.6 mm in diameter) into smaller particles, which were then ingested. Within 4 h, 80–100% of the feed was ingested, corresponding to 17 ± 8 mg feed h⁻¹ per shrimp (mean ± SD; n = 5; one nonfeeding shrimp excluded). Gut passage time was 35 ± 5 min (n = 6). Feces egested within 4 h accounted for 20% of the ingested feed (3 ± 1 mg feces h⁻¹ per shrimp; see Fig. 1 for mass balance). Thus, ingestion and egestion equaled 2.4% and 0.4%, respectively, of shrimp body weight. Feces were produced as a continuous fecal strand ∼1–2 mm thick, surrounded by a peritrophic membrane. The dry weight of feces and feed was 18 ± 1% and 92 ± 1%, respectively.

**Microbial abundance**

The bacterial density in fresh feces ranged from 0.6–6.6 × 10^{10} cells g⁻¹ dry wt in Experiments A, B, and C. These
bacteria were derived from the shrimp gut and their numbers increased by ~3–9-fold within 12 h postegestion (Fig. 2). Their assemblage-average net generation time thus equaled 6.3 ± 2.5 h (range = 4–8 h) or a minimum net growth rate of 1.9–4.3 day⁻¹. In the incubation water, bacterial abundance increased rapidly during 12 h from zero to 1.3 × 10¹⁰ cells L⁻¹ (Experiment A), 6.7 × 10¹⁰ cells L⁻¹ (Experiment B), and 5.9 × 10⁹ cells L⁻¹ (Experiment C). Bacterial net production (increase in abundance) was 1.6 × 10¹¹ and 1.7 × 10¹¹ cells g⁻¹ dry wt feces per 12 h in Experiments A and B, but much lower in Experiment C (2.3 × 10⁹ cells g⁻¹ dry wt feces per 12 h), corresponding with lower bacterial abundance in the feces at t₀ in Experiment C. Bacterial abundance in RAS water was 2.8 × 10¹¹ ± 1.7 × 10¹⁰ cells L⁻¹

Despite careful microscopic examination, protists could not be detected in the feces at any time.

**Bacterial cell size and biomass**

Bacteria in fresh feces were large (average, 0.77 ± 0.71 μm³) and their size decreased significantly within 12 h to 0.37 ± 0.34 μm³ (P < 0.001) and 0.41 ± 0.41 μm³ (P < 0.001) in feces and incubation water, respectively (Experiment A, Table 1). The decrease was probably due to rapid growth, consistent with the presence of large numbers of smaller, probably recently divided cells. However, Gammaproteobacteria of a conspicuously large morphotype (below), originally smaller than the community average (distinguished by their shape), more than doubled in size within 12 h in feces and seawater (both P < 0.05; Table 1). Yet, they grew slowly, yielding a low increase in abundance.

Cellular protein content ranged from 49–108 fg per cell (Table 1) and the total bacterial protein in feces increased from 0.5% to 1.8% in 12 h (Experiment A; Table 1). The total bacterial biomass in feces increased from 2.9 to 16.8 mg g⁻¹ dry wt within 12 h; thus, bacterial biomass comprised 0.3% (t₀) and 1.7% (t₁₂h) of fecal dry matter. This sixfold increase in biomass corresponded to a ninefold increase in bacterial abundance in the feces. Bacterial biomass in the incubation water developed at 12 h to 1.2 mg L⁻¹.

**Bacterial production**

The bacterial production rate in freshly released feces was high (1.9 × 10¹⁰ cells g⁻¹ dry wt h⁻¹), but decreased > 5-fold at t₁₂h (3.5 × 10⁹ cells g⁻¹ dry wt h⁻¹). Production in the incubation water at t₁₂h was 3.1 × 10⁹ cells L⁻¹ h⁻¹ (Table 2), also much higher than that in the RAS water (7.9 × 10⁸ cells L⁻¹ h⁻¹).

**Bacterial community composition**

In Experiment A, 64–77% of bacteria in feces and the incubation water were Bacteroidetes, Alphaproteobacteria, and Gammaproteobacteria (Fig. 3). The mean detection rate with probes EU338 I–III was 94% of all DAPI-stained cells and Betaproteobacteria always contributed <1% of the DAPI-counts. Gammaproteobacteria were always dominant.

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**Table 1. Mean (± SD) of bacterial cell size (three replicates with 50–100 cells measured, each), calculated cell volume, protein content, and biomass from Experiment A**

<table>
<thead>
<tr>
<th>Time</th>
<th>Length (μm)</th>
<th>Width (μm)</th>
<th>Cell volume (μm³)</th>
<th>Total bacterial biomass (mm² g⁻¹ dry feces or mm³ L⁻¹)</th>
<th>Protein/ cell* (fg per cell)</th>
<th>Total bacterial protein (mg g⁻¹ dry feces or mg L⁻¹)</th>
<th>Total bacterial dry biomass¹ (mg g⁻¹ dry feces or mg L⁻¹)</th>
<th>Bacterial protein (% of fecal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t₀</td>
<td>1.71 (0.63)</td>
<td>0.83 (0.26)</td>
<td>0.77 (+0.71)</td>
<td>14.81 (+15.35)</td>
<td>75.7</td>
<td>1.46</td>
<td>2.93</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Feces¹</td>
<td>Large Gammaproteobacteria</td>
<td>3.86 (2.25)</td>
<td>0.47 (0.13)</td>
<td>0.64 (+0.70)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>t₁₂h</td>
<td>1.37 (0.46)</td>
<td>0.64 (0.21)</td>
<td>0.37 (+0.34)</td>
<td>63.61 (+62.57)</td>
<td>49.2</td>
<td>8.38</td>
<td>16.77</td>
</tr>
<tr>
<td></td>
<td>Feces¹</td>
<td>Large Gammaproteobacteria</td>
<td>5.23 (2.46)</td>
<td>0.59 (0.15)</td>
<td>1.40 (+1.34)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>t₁₂h</td>
<td>1.37 (0.49)</td>
<td>0.67 (0.25)</td>
<td>0.40 (+0.41)</td>
<td>4.83 (+5.32)</td>
<td>51.9</td>
<td>0.62</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>Seawater¹</td>
<td>Large Gammaproteobacteria</td>
<td>4.71 (1.55)</td>
<td>0.56 (0.09)</td>
<td>1.10 (+0.71)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Determined according to Simon & Azam (1989): y = 88.6 × x⁰.⁵⁹.

¹Based on bacterial protein content of 50%.

²Without large Gammaproteobacteria when separate values are given.
respectively, or growth rates of 5.5, 6.4, 3.9, and 3.2 day

Given that bacterial mortality was low, we estimate the
as fast as

Alphaproteobacteria

group to the community indicated that
abundance, the change in the relative contribution of each
oidetes

were equally abundant after 12 h.

In freshly released feces, statistically, all Gammaproteobacteria
belonged to the genus Vibrio (Fig. 3). However, one large
morphotype hybridizing with the gammaproteobacterial
probe, but not with the Vibrio-specific probe, contributed
9.6 × 10⁷ cells g⁻¹ dry wt, 1.2 × 10⁹ cells g⁻¹ dry wt, and
3.6 × 10⁷ cells L⁻¹ to bacterial abundance in the feces (t₀ and
12h), and in the incubation water (t₁₂h), respectively (direct
DAPICounts, distinguished by morphology). In the incubation
water, Vibrio spp. and members of the phylum Bacteroidetes
were equally abundant after 12 h.

Although all phylogenetic groups analyzed increased in
abundance, the change in the relative contribution of each
group to the community indicated that Bacteroidetes and
Alphaproteobacteria in the feces would have grown about twice
as fast as Gammaproteobacteria and their subgroup Vibrio spp.
Given that bacterial mortality was low, we estimate the
generation times of Bacteroidetes, Alphaproteobacteria, Gam-
maproteobacteria, and Vibrio spp. to be 3.0, 2.6, 4.2, and 5.3 h,
respectively, or growth rates of 5.5, 6.4, 3.9, and 3.2 day⁻¹.

Bacteria in the nearly empty guts of sacrificed shrimp consisted of 21% Bacteroidetes, 5% Alphaproteobacteria, and

5% Vibrio spp. of DAPI-Counts (40% detection rate with
EUB probes). Bacteria in RAS water (54% EUB-detection
rate) were 6% Bacteroidetes, 7% Gammaproteobacteria, and
4% Vibrio spp. of DAPI-Counts.

Hydrolitic ectoenzyme activity

Enzyme activities were always very high, ranging from 0.1 to
42μM h⁻¹ of fluorogenic substrate hydrolyzed (Figs 4–6). In
the feces, alkaline phosphatase and N-acetylgalcosaminidase
activities were the highest, followed by aminopeptidase
activity. Over the incubation period, most enzyme activities
increased significantly. Aminopeptidase activity in the feces
was bacterial).

In the RAS water, aminopeptidase activity was the
highest (5.9 ± 0.3μM h⁻¹), followed by β-glucosidase

Table 2. Total bacterial production in feces and the incubation seawater (H₂O) in Experiment A calculated from BrdU incorporation rates (BrdU+ cells) and as net production from differences in abundances.

<table>
<thead>
<tr>
<th></th>
<th>BrdU+ cells g⁻¹ dry wt h⁻¹</th>
<th>BrdU+ cells g⁻¹ dry wt per 12 h</th>
<th>Bacterial abundance* (t₁₂h – t₀)</th>
<th>Net bacterial production (t₁₂h – t₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces t₀</td>
<td>1.9 × 10¹</td>
<td>2.3 × 10¹</td>
<td>1.9 × 10¹</td>
<td>–</td>
</tr>
<tr>
<td>Feces t₁₂h</td>
<td>3.5 × 10⁹</td>
<td>4.2 × 10⁹</td>
<td>1.7 × 10¹</td>
<td>1.5 × 10¹</td>
</tr>
<tr>
<td>H₂O t₁₂h</td>
<td>3.1 × 10⁹</td>
<td>3.7 × 10¹</td>
<td>1.2 × 10¹</td>
<td>1.2 × 10¹</td>
</tr>
</tbody>
</table>

*Cells g⁻¹ dry wt for feces, and cells L⁻¹ for H₂O.

1BrdU+ cells L⁻¹ h⁻¹.

2BrdU+ cells L⁻¹ per 12 h.
activity (0.8 ± 0.01 μM h⁻¹), which is in the same range as in
the incubation experiments. However, alkaline phosphatase
activity in the RAS was 0.3 ± 0.06 μM h⁻¹ and thus an
order of magnitude lower than that in the incubation
experiments.

**Organic C, N, and protein contents of the feed**

Feed comprised 41% TOC and 6% TON. The feed C/N ratio
was 6.9 and the protein content was 35.4 ± 1.3%. After a 12-
h exposure to sterile, particle-free seawater, the C concen-
tration of the recovered feed did not change significantly,
whereas its N concentration increased by 10% (C/N of 6.6;
Table 3). The incubation seawater became enriched in
dissolved C and N due to release from the feed (DOC-
53 ± 0.6 mg g⁻¹ dry feed; DON = 0.7 ± 0.1 mg g⁻¹ dry feed;
Fig. 7). Most of this release (82%) occurred within 4h. The
release of fine POC and PON from the feed into the seawater
within 12 h was 3.2 ± 0.03 mg g⁻¹ dry feed and 0.4 ± 0.01 mg g⁻¹ dry feed, respectively (sum of TOC and
Table 3). Thus, C and N accumulation in the water
accounted for a loss of 14% C and 2% N from the feed,
indicating a better retention of N in feed. Because the
amount of TOC per gram feed did not change over time,
this C loss also corresponded to a total loss of dry feed of
14% (Table 3 and Fig. 1).

**Table 3. C and N budget in feed (n = 3) and feces (n = 7) incubated in
sterile seawater for 12 h**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time (h)</th>
<th>TOC (mg g⁻¹ dry wt)</th>
<th>C/N</th>
<th>TON (mg g⁻¹ dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed</td>
<td>0</td>
<td>6.9 (± 1.0)</td>
<td>407 (± 25)</td>
<td>59 (± 1)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>6.6 (± 0.1)</td>
<td>428 (± 13)</td>
<td>65 (± 2)</td>
</tr>
<tr>
<td>Released</td>
<td>12</td>
<td>6.5 (± 1.1)</td>
<td>56 (± 1)</td>
<td>1.1 (± 0.1)</td>
</tr>
<tr>
<td>Feces</td>
<td>0</td>
<td>6.4 (± 0.6)</td>
<td>375 (± 45)</td>
<td>59 (± 11)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>6.4 (± 0.8)</td>
<td>358 (± 51)</td>
<td>56 (± 9)</td>
</tr>
<tr>
<td>Released</td>
<td>12</td>
<td>4.2 (± 0.4)</td>
<td>101 (± 16)</td>
<td>5.1 (± 0.8)</td>
</tr>
</tbody>
</table>

Mean (± SD).

*POC/PON of seawater after 12 h (C/N of seawater at t₀ = 6.5 ± 1).
'forty = 3.
'PON, not TON.

**Organic C, N, and protein contents of the feces**

Freshly produced feces contained 38% TOC and 6% TON,
and a C/N ratio of 6.4 (Table 3). The protein content of the
feces ranged from 28–45% (Experiments A and C; Fig. 8).
When feces were exposed to sterile, particle-free seawater for
12 h, the TOC and TON contents did not change significantly (Table 3).
However, the PON composition changed,
reflected in a 68% and 18% increase in the protein concen-
tration in Experiments A and C, respectively (Fig. 8).
Although the protein concentrations of freshly released feces
varied between experiments, they increased significantly in
each experiment (Experiment A: α: P < 0.05, and C: P < 0.05), with larger increases corresponding to lower
protein concentrations and higher bacterial abundance at
t₀. During 12 h, the feces released into the incubation water
78 ± 15 mg g⁻¹ dry wt DOC (Fig. 7), 23 ± 5 mg g⁻¹ dry wt
POC, and 5 ± 1 mg g⁻¹ dry wt PON (Table 3; DON was not
measured in these samples). The total release of organic
matter from the feces was equivalent to 27%. However, the release of DOC from the feces was much slower (3% and 9% after 4 and 8 h, respectively) than from the feed.

Analysis of feces from individual shrimp (Experiment C) revealed large differences in their TOC and TON contents. Fecal TOC and TON corresponded with the individual shrimp’s feeding activity; feeding shrimp released feces of high TOC and TON contents, whereas weakly or nonfeeding shrimp produced feces of significantly lower TOC and TON values (Table 4).

### Discussion

#### Feed processing

In intensive *P. vannamei* RAS, feed is provided *ad libitum* and feces are produced rapidly. In this study, gut passage time was 30–60% shorter than previous reports for similar-sized *P. vannamei* or *Penaeus (Farfantepenaeus) aztecus* (Beseres et al., 2006). Unless removed by mechanical filtration, feces and excess feed would remain in RAS for extended time periods, thus enriching the RAS water with DOM and POM. We found a minimum loss of 14% of input feed within 12 h even though shrimp were not fed excessively. Feces and feed bioturbation and mastication by shrimp likely expedite dissolution and disintegration, channeling substantial nutrients and energy to the microbial loop.

#### Bacterial abundance, production, and turnover rates

Freshly produced shrimp feces harbored highly abundant and active bacteria. Comparisons of our direct bacterial counts in shrimp feces with published values are difficult because previous studies used culture-based methods. However, bacterial abundances in the gut and feces of *P. vannamei* were of the same order of magnitude as in Moss et al. (2000); they estimated $2 \times 10^{10}$ CFU g$^{-1}$ gut tissue in *P. vannamei*. Shrimp in our study were kept in autoclaved seawater and fed autoclaved feed and this should have minimized contamination. Therefore, the likely source of the feces-derived bacteria was the *P. vannamei* gut microbiome. These bacteria grew rapidly in egested feces and quickly colonized the incubation water.

Bacterial production in shrimp feces was measured using the BrdU method, and independent minimum estimates were obtained by calculating net increase in bacterial cell abundance over time. As expected, the net increase in bacterial abundance in the feces was lower than that predicted from BrdU incorporation rates at $t_0$, but only by 30%. BrdU-based bacteria production was likely overestimated because the $t_0$ rate was assumed to be steady throughout the 12-h incubation (but it actually decreased). Thus, the finding of comparable BrdU-based bacteria production and cell-abundance-based net bacteria production, on face value, would suggest that bacterial mortality, for example by grazing and phage, was a minor factor in assemblage dynamics. This observation is consistent with the lack of protists in the feces. Bacterial mortality is likely to be greater in the entire RAS due to its greater trophic complexity.

Bacterial abundance and production in the RAS were similar (Moriarty, 1986) or one to two orders of magnitude lower (Allan et al., 1995) than in open pond systems for penaeid shrimp and very similar bacterial abundances were reported for shrimp RAS with very high stocking densities (Otoshi et al., 2009). The bacterial assemblage generation time in our RAS (10 days) was 3–10-fold slower than in Allan et al. (1995). Some of the difference may be due to the different conversion factors used to calculate cell production. We used a cell-size-dependent conversion factor (Simon & Azam, 1989) because bacteria were larger (higher cell C) than those in Moriarty (1986) and Allan et al. (1995). However, another study from an open system (but with lower shrimp stocking density) showed that bacterial abundance and production in different ponds operated under the same conditions may vary by three orders of magnitude (Alongi et al., 1999). The mean values from this system were comparable to our RAS. High variability in bacteria carbon demand has implications for carbon flow pathways and variability in system function.

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**Table 4.** C and N content of feces from individual shrimp

<table>
<thead>
<tr>
<th>Shrimp #</th>
<th>Feeding activity</th>
<th>C/N</th>
<th>Carbon (mg g$^{-1}$ dry wt)</th>
<th>Nitrogen (mg g$^{-1}$ dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No</td>
<td>4.8</td>
<td>163</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>6.4</td>
<td>449</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>7.7</td>
<td>516</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>5.9</td>
<td>476</td>
<td>81</td>
</tr>
<tr>
<td>5</td>
<td>Medium</td>
<td>5.4</td>
<td>392</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>No</td>
<td>7.3</td>
<td>324</td>
<td>45</td>
</tr>
</tbody>
</table>

**Fig. 8.** Protein content in feed and feces as percent of dry weight (mean ± SD; n = 3) at $t_0$ and $t_{12h}$.
Bacterial population turnover time (abundance/production) was drastically faster in shrimp feces (1–10 h) than in mature RAS water (350 h). Extrapolating these results to RAS operated at our experimental site (2.6 × 10⁷ shrimp at 18 g; 58 m² RAS surface area; 1 m depth; 2 kg feces produced day⁻¹; using same shrimp stock and feed as in our microcosms), comparable amounts of new bacteria would be produced in the feces (∼9 × 10¹⁴ cells day⁻¹) as in the entire RAS water (∼11 × 10¹⁴ cells day⁻¹). Thus, shrimp feces are hotspots of intense bacterial growth and production in the RAS. Shrimp gut-derived bacteria might not only ‘inoculate’ RAS water but may also contribute to bacterial abundance and productivity budgets. These hotspots, produced necessarily during the RAS operation, may also act as loci of explosive growth of shrimp pathogenic bacteria.

**Bacterial community composition**

The high detection rate of bacteria in feces and incubation water with the EUB probes (94%) is probably due to the large cell size and ribosomal content, whereas the low detection rate in the gut (40%) corresponded with smaller (possibly inactive) cells. This could be due to food limitation before shrimp immolation and/or the degradation of gut bacteria by shrimp enzymes. In all environments, 64–77% of DAPI-stained cells (equivalent to 69–79% of EUB-positive cells) belonged to the phylotypes *Bacteroidetes*, *Alphaproteobacteria*, and *Gammaproteobacteria*, but it is not known whether the differences in the relative abundances of these phylotypes in the feces and gut were due to differential survival (e.g. by attachment) of the bacteria or preferential digestion by the shrimp. The ∼20–30% of EUB-positive cells not accounted for with the probes used could belong to other phylogenetic groups or escaped detection due to the lower group coverage of some of the probes (Aman & Fuchs, 2008).

This culture-independent analysis of bacterial composition in shrimp feces corroborates earlier studies in which members of the above phylogenetic groups (particularly *Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp., *Beneckia* spp., *Xanthomonas* spp., *Alteromonas* spp., *Photobacterium* spp., *Alcaligenes* spp., *Cytophaga* spp., and *Flavobacterium* spp.) were frequently isolated from the guts of different shrimp species from aquaculture systems and natural habitats (Dempsey & Kitting, 1987; Harris, 1993; Gomez-Gil et al., 1998; Moss et al., 2000). The apparently low diversity in the shrimp gut and the dominance of *Vibrio*-like bacteria is consistent with a limited number of sequences recovered in 16S rRNA gene clone libraries from the gut flora of *P. vannamei* and other shrimp (Lau et al., 2002; Johnson et al., 2008). We found that almost all *Gammaproteobacteria* identified in freshly released feces belonged to the genus *Vibrio*. Although some vibrios are fish/shellfish pathogens, those that we detected in feces were likely nonpathogenic because we used only healthy shrimp with no signs of vibriosis.

Because many aerobic marine *Gammaproteobacteria*, *Alphaproteobacteria*, and *Bacteroidetes* also grow under anoxic conditions (Alonso & Pernthaler, 2005), they might survive the transition from seawater to shrimp gut and via the feces back into seawater, meanwhile degrading chitinous and proteinaceous shrimp ingesta as well as the chitinous peritrophic membrane of the feces. These bacteria could compete with shrimp for nutrients, but their enzymes could also aid shrimp in the digestion of complex substrates (Wainwright & Mann, 1982) in addition to serving as direct food for the shrimp (Moss, 2002). Further, their degradation of the peritrophic membrane could disintegrate the feces, with consequences for the system.

In agreement with studies from other environments (Yokokawa et al., 2004; Tada et al., 2010), we found that bacterial production differed among phylotypes. This was especially shown in the differences in community composition between feces and incubation water after 12 h and implied that bacteria were not exclusively randomly dislodged from the feces, but that they grew in situ in the seawater. Cottrell & Kirchman (2000) demonstrated variations in phylotype-specific utilization patterns for different (including simple low-molecular-weight) DOM components, but that no single phylogenetic group dominated the consumption of all substrates. They suggest that a diverse microbial community is essential for the complete degradation of complex DOM. This is relevant to the ability of bacteria to compete with shrimp for organic matter as well as potentially enrich refractory POM with nutritious bacterial biomass.

**Enzymatic hydrolysis of organic matter**

Bacterial cell surface hydrolases are important in polymer and particle hydrolysis. Thus, analyzing enzyme activities in the feces should help elucidate the links between bacterial community structure and function. We found very high activities for all measured enzymes. Alkaline phosphatase, aminopeptidase, and N-acetylglucosaminidase were active at μM h⁻¹ in the feces, incubation water, and RAS water, indicating the ability to degrade compositionally diverse organic matter. β-Glucosidase, α-glucosidase, and lipase activities were comparatively low, but still an order of magnitude higher than in coastal oceans (Riemann et al., 2000; Zoppini et al., 2005). Although β-glucosidase activity increased significantly in feces and seawater during the incubation, it remained well below phosphatase and peptidase activities. Relatively slower hydrolysis of polysaccharides would cause accumulation of refractory organic carbon in the system, while protein and chitin would be used rapidly by bacteria.
Not all hydrolase activity in the feces was necessarily bacterial; *P. vannamei* digestive enzymes most likely made a contribution. While we could not separate the two enzyme sources, we note that maximum cell-specific activities (computed by assuming all enzyme activities to be bacterial) were comparable to those from marine environments (e.g. Riemann et al., 2000). Further, the enzyme activities increased substantially during incubation presumably due to bacterial growth and enzyme production. We therefore think that bacteria contributed significantly to the high hydrolase activity in our incubation experiments and in the RAS. Because bacteria-mediated POM → DOM transition is important in flux partitioning between the microbial loop and shrimp, future studies should better characterize the enzymes and their regulation.

**Organic C, N, and protein**

In our incubation experiments, shrimp feces showed a significant increase in the protein concentration. A 40–85-fold increase in the protein content caused by externally colonizing bacteria has been reported for mollusc feces (Newell, 1965). In our study, despite high bacterial biomass developing in the feces, bacterial protein constituted only a few percent of the total protein in the feces and thus bacterial biomass alone could not explain the increase in the protein content. Intriguingly, the increase in the protein content in 12-h incubation is not accompanied by changes in the total C and N concentrations in the feces. Despite careful scrutiny, we could not find any measurement error. However, part of the measured protein could have been ammonium or other primary amines (developed, e.g., during the microbial degradation of organic, nonprotein N-compounds) that can be readily incorporated into bacterial biomass. With respect to these uncertainties, we further discuss only the observations that freshly egested feces were bacteria and protein rich and that these bacteria continued to metabolize and grow in the feces, enriching it with respect to bacterial biomass and possibly additional protein.

Shrimp fed excess feed likely perform superfluous feeding (e.g. Gleason, 1986); consequently, large amounts of undigested or partially digested feed are egested. In the digestive system of (coprophagous) rodents, conditioning of the feces via bacterial synthesis of nutrients occurs in the lower gastrointestinal tract, where little absorption is realized, and subsequent coprophagy provides a mechanism for obtaining these nutrients (Soave & Brand, 1991). Microbial conditioning from the inside as well as from colonization from outside of the fecal pellets has also been described for feces from aquatic animals (Lawrence et al., 1993; Wotton & Malmqvist, 2001) and various shrimp species are known to be coprophagous (Frankenberg & Smith, 1967; Rothans & Miller, 1991). Our incubation experiments suggest the hypothesis (dubbed ‘gut extension hypothesis’) that shrimp admixes undigested food with gut bacteria and shrimp digestive hydrolases to high levels; hence, the egested fecal organic matter continues to be digested as well as enriched via bacterial growth – until ingested by shrimp. A prediction would be that the organic matter in aged feces is more readily digestible to the shrimp and could serve as an important feed source. This may be an adaptation of shrimp in their natural environment, where feed can be scarce, of low nutritional quality, and not readily digestible. In an RAS operation, reducing the feeding rate might enhance coprophagy and lead to improved water quality and reduced feed cost.

**Conclusion**

While the scope of this research was limited, the results from the approach adopted here underscore the importance of microbial processes in RAS functioning. We have demonstrated that diverse and abundant bacteria egested into the feces by shrimp have the potential for explosive growth. These bacteria could nutritionally enrich the feces, but future studies should also consider whether (and under what conditions) the feces might serve as a vector for the proliferation of shrimp pathogens (which could infect shrimp through coprophagy). The ecosystem approach could also help elucidate ecosystem conditions that lead to the proliferation of functionally critical bacteria (e.g. nitrifiers that mitigate nitrite and ammonia accumulation to levels toxic to shrimp). Broadly, we need an integrative and mechanistic view of RAS as controlled ecosystems in which – because of intensive feeding regimens – bacteria play major roles in elemental fluxes and organic matter processing. Such an approach could facilitate predictions on system health and stability, improve system management, and help develop sustainable aquaculture.

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