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Copepod guts as biogeochemical hotspots in the sea: Evidence from microelectrode profiling of *Calanus* spp.

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Abstract

The environmental conditions inside the gut of *Calanus hyperboreus* and *C. glacialis* were measured with microelectrodes. An acidic potential hydrogen (pH) gradient was present in the gut of *C. hyperboreus*, and the lowest pH recorded was 5.40. The gut pH of a starved copepod decreased by 0.53 after the copepod resumed feeding for a few hours, indicating the secretion of acidic digestive fluid. A copepod feeding on *Thalassiosira weissflogii* (diatom) had slightly lower pH than that feeding on *Rhodomonas salina* (cryptophyte). Oxygen was undersaturated in the gut of both *C. hyperboreus* and *C. glacialis*, with a steep gradient from the anal opening to the metasome region. The central metasome region was completely anoxic. Food remains in the gut led to a lower oxygen level, and a diatom diet induced a stronger oxygen gradient than a cryptophyte diet. The acidic and suboxic–anoxic environments of the copepod gut may support iron dissolution and anaerobic microbial activities that otherwise are not favored in the well-buffered and oxygenated ambient ocean.

Copepods are the most abundant metazoans in the ocean (Verity and Smetacek 1996). By consuming phytoplankton, microzooplankton, and detritus, they process a large percentage of the suspended particulate matters in the water column (Heinle et al. 1977; Gifford 1991; Calbet 2001). From ingestion to digestion and defecation, particulate matter is transformed physically and biochemically by these consumers, the outcome of which has significant ramifications for energy and material fluxes in the ocean (Cowie and Hedges 1996; Turner 2002). Both microscopy and bioassays have shown that a copepod's gut is heavily colonized by microbes, whose activities may augment that of the copepod in transforming materials that pass through the gut (Tang et al. 2010). To fully understand the roles of copepods in global biogeochemical cycles, we need a better understanding of the environmental conditions of copepod guts where the ingested materials are being processed.

It has long been speculated that a copepod gut is acidic and a hotspot for carbonate dissolution in the ocean (Harris 1994; Milliman et al. 1999; Jansen and Wolf-Gladrow 2001). To our knowledge, direct in vivo measurement of copepod gut potential hydrogen (pH) has been attempted only once using a pH-sensitive dye, which showed that the gut of starved *Calanus helgolandicus* was slightly acidic (median pH = 6.86–7.19; minimum = 6.11) relative to seawater (Pond et al. 1995). The resolution of this pH-sensitive dye is, however, rather limited, and it does not allow for a more detailed characterization of the gut pH. Researchers have also long speculated that copepod gut may be suboxic to anoxic and, therefore, support anaerobic microbial activities. Circumstantial evidence includes the presence of strict anaerobes, such as methanogens (Marty 1993) and nitrogen fixers (Proctor 1997; Braun et al. 1999), associated with copepods. Because many critical microbial processes preferably occur in suboxic or anoxic condition, the question of whether a copepod's gut is suboxic or anoxic has profound implications for global biogeochemistry. For example, the potent greenhouse gas methane can be produced by microbes primarily under anoxic conditions (but *see* Karl et al. 2008). A well-known paradoxical phenomenon is methane oversaturation in a well-oxygenated mid-water column (Reeburgh 2007). Many researchers have hypothesized that zooplankton gut is the source of this mid-water methane, and it has been shown that methane is produced when zooplankton actively graze on phytoplankton (DeAngelis and Lee 1994). However, in the absence of direct oxygen measurements, it remains debatable if a zooplankton gut can be anoxic and support methanogenesis (Conrad 2009).

Oxygen and pH microelectrodes have been widely used for studies of benthic biogeochemistry and photosynthesis (Kühl and Revsbech 2001; Glud 2008). The tip region of such sensors can be made as thin as only a few micrometers, which makes them applicable for characterizing conditions in microaggregates or digestive tract of invertebrates with minimal disturbance (Brune and Kühl 1996; Ploug et al. 2002). In this study, we took advantage of this technology and directly measured the pH and oxygen inside the gut of living and feeding copepods. The implication of our data is discussed in the context of acidic and anaerobic biogeochemical processes in the otherwise well-buffered and -oxygenated pelagic ocean.

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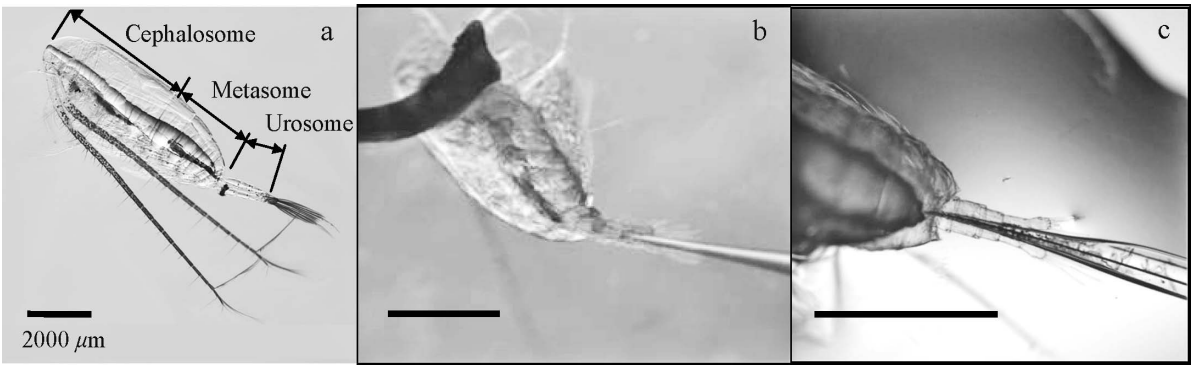

Fig. 1. (a) *Calanus hyperboreus* showing the different gut regions as defined in this study. (b) Direct insertion of a pH microelectrode through the anal opening of a tethered *C. hyperboreus*. (c) An oxygen microelectrode reaching the metasome region. All copepods were alive and in some experiments actively feeding (*see* text). All scale bars are 2000 μm. Photo credit for Fig. 1a: Russ Hopcroft, University of Alaska Fairbanks.

Methods

Because a copepod has a relatively straight gut and unobstructed anal opening (compared to, for example, a daphnid), it is possible to immobilize a live copepod and directly insert a microelectrode through the anal opening. Based on morphology and cell types, a copepod's gut can be divided into (roughly) three sections (Mauchline 1998): foregut, midgut, and hindgut, although the distinction between sections is somewhat arbitrary, and may vary from species to species or even from study to study (Mauchline 1998). Because our focus was the chemical conditions inside the gut rather than the different morphology and cell types, we divided the gut into three regions (Fig. 1a): the cephalosome region that extended from the oral opening to the end of the cephalosome segments, the metasome region that spanned across the metasome segments, and the urosome region within the urosome segments ending in the anal opening. The size of the microelectrode tip relative to a copepod necessitates the use of large copepod species. We, therefore, chose the large *Calanus* species (*C. hyperboreus* and *C. glacialis*) that dominate the zooplankton communities in arctic and subarctic waters.

Experiments were conducted in May of 2009. *Calanus hyperboreus* were collected on board R/V *Porsild* (Arctic Station, University of Copenhagen) at a monitoring station in Disko Bay, western Greenland (69°14′N, 53°23′W [Nielsen and Hansen 1995; Madsen et al. 2001]). *C. glacialis* were collected from the central part of Godthåbsfjord near Nuuk (sta. GF7 in Arendt et al. 2010). In both cases, the animals were collected by vertical hauls within 0–100 m using a 200-μm Working Party 2 (WP-2) net (UNESCO 1968) equipped with a nonfiltering cod end. All animals were transported back to the Greenland Climate Research Center in Nuuk (64°10′N, 51°44′W), and maintained in natural seawater inside an environmental room (5.5 ± 0.2°C). In situ temperature at the Nuuk location is 3–6°C in the upper water layers during May (Mortensen et al. 2011). Only live copepodites of stage 4–5 were used for the experiments. For the different diet treatments, the copepods were fed either the cryptophyte *Rhodomonas salina* or the diatom *Thalassiosira weissflogii* in exponential growth phase, or in some cases nothing (GF/F-filtered seawater), for > 24 h prior to measurements.

For microprofiling, a live copepod was selected and transferred onto a glass slide using a wide-mouth pipette. The surrounding water was then siphoned away as much as possible without harming the animal. A thin nylon thread was quickly attached to the dorsal side of the cephalosome or metasome with a small drop of superglue. The tethered copepod was then moved to a petri dish with seawater and observed under a dissecting microscope. After it was confirmed that the tethered copepod was alive and unharmed, the nylon thread holding the copepod was fastened to a lump of submerged wax at the base of a petri dish. The petri dish was then put inside a small aquarium connected to a circulating water bath (6–7°C, comparable to in situ summer surface temperature; Mortensen et al. 2011). The water in the water bath was kept fully air-saturated by an air pump. Further, a small glass tube directed a continuous airstream at the water film in front of the tethered animal to ensure a stable and unidirectional water flow around the animal. Microelectrodes were manually controlled by a micromanipulator and directed toward the anal area using a stereo microscope (Fig. 1b,c). The sensors were moved in steps of 50 μm or 100 μm, and sensor signals were recorded at each step. A microprofile typically took 15–25 min to complete after the first measurement. The size of the microelectrodes limited our measurements to the urosome region and part of the metasome region. Care was taken to ensure that the electrode was positioned inside the gut during the profiling. In the cases where the electrode accidentally punctured the gut epithelium, the profile was discarded.

pH microprofiles were measured using Lix-electrodes with a tip diameter of 1–2 μm and a response time of 10–20 s (De Beer et al. 1997). The sensor signal was recorded by a millivoltmeter (Pyro-science MV600) connected to a strip-chart recorder. Prior to experiments, the sensors were calibrated using standard buffers at the experimental temperature.

Oxygen profiles were measured with a Clark-type microelectrode equipped with a guard cathode and an internal reference (Revsbech 1989). The tip diameters were 1–3 μm, stirring sensitivity was < 1% and the 90% response

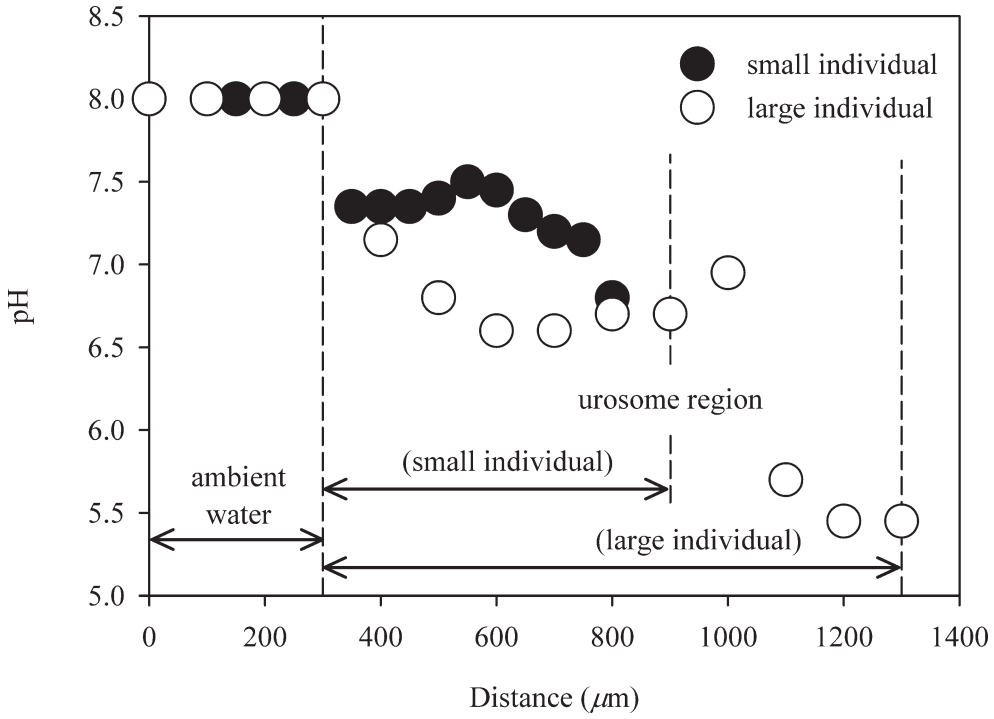

Fig. 2. Gut pH profiles of *Calanus hyperboreus* (n = 2) that had been feeding on *Thalassiosira weissflogii*. Distance was measured from ambient water into the gut. The dashed lines indicate the positions of the anal opening and the approximate urosome–metasome transition.

time was < 2 s (Gundersen et al. 1998; Glud et al. 2000). The sensor current was measured by a picoammeter (Unisense PA2000) connected to an analog-to-digital converter, which transferred the signal to a computer and a strip-chart recorder (Revsbech and Jørgensen 1986). The microelectrodes had a linear response, and were calibrated prior to the experiments at the experimental temperature by successively inserting the sensor tip into samples of 100% air-saturated and anoxic seawater. Anoxic seawater for calibration was produced by flushing with N_2 and adding dithionate to chemically remove any trace amount of O_2.

Results

pH profiles—Seven individuals of *C. hyperboreus* were successfully profiled for their gut pH. The gut pH of a smaller individual that had been feeding on *T. weissflogii* was measured as the microelectrode was moved from ambient water into the gut (Fig. 2). As the electrode moved past the anal opening, the pH quickly dropped from 8.00 (ambient water) to 7.35. The pH remained rather stable within the first 250 μm, then dropped to 6.80 as the electrode moved closer to the metasome region (but could not go further). In the second trial with a larger individual, we inserted the electrode to the head of the urosome region and measured the pH as the electrode was moved outward (Fig. 2). At the head of the urosome region, the pH was 5.45, clearly indicating acidic condition. pH increased quickly as the electrode reached approximately the inner one-quarter of the distance, and stayed relatively constant at around 6.70 ± 0.10 until near the anal opening, where the pH increased to the ambient value. In contrast to the O_2 microprofiles (*see* below), it was difficult to obtain continuous pH profiles due to sensor breakage, but several microprofile segments confirmed this overall pattern.

Additional experiments were conducted to test whether the copepod's feeding history influenced the gut pH (Table 1). To avoid breakage, the sensor was only inserted to the central part of the urosome or metasome regions.

Table 1. Gut pH of *Calanus hyperboreus* under different diet treatments. The microelectrode was positioned at approximately half-way up the urosome or the metasome region. In some cases, multiple measurements were made on a single copepod. One of the starved individuals was fed *Rhodomonas salina* for a few hours and remeasured for its metasome region pH. Ambient water temperature was 6.5°C and ambient water pH was 8.00.

Individual	Diet type	Gut region	pH
1	*Rhodomonas salina*	urosome	6.70
		metasome	6.42
2	*Thalassiosira weissflogii*	urosome	6.14
			5.40
			5.44
3	starved	urosome	6.20
		metasome	6.24
4	starved	urosome	6.47
		metasome	6.44
5	starved	metasome	6.34
			6.32
	resumed feeding	metasome	5.80

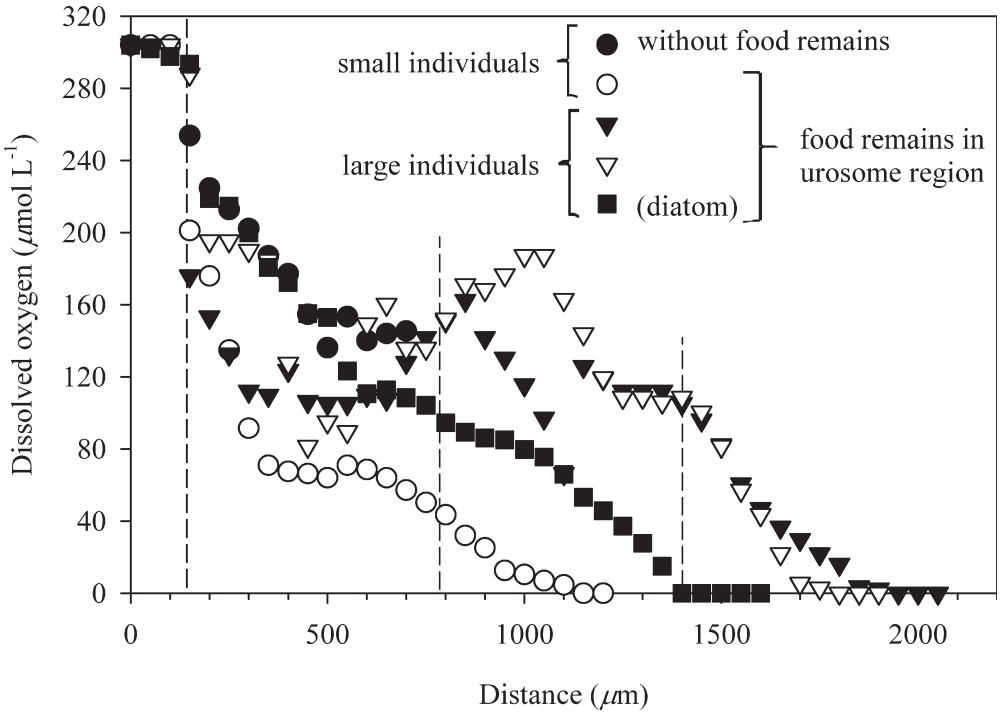

Fig. 3. Gut oxygen profiles of *Calanus hyperboreus* (n = 5) that had been feeding on *Rhodomonas salina* or *Thalassiosira weissflogii* (diatom), without or with visible food remains in the gut. Distance was measured from ambient water into the gut. The left dashed line indicates the position of the anal opening. The middle and right dashed lines indicate the approximate positions of urosome–metasome transition for the small and large individuals, respectively. Ambient temperature was 7°C and salinity 34; 100% dissolved oxygen saturation was at 304 μmol L^{-1}.

For starved copepods, the pH in metasome and urosome regions was 6.20–6.47, still lower than ambient water, and was similar to the one that had been feeding on *R. salina* (metasome = 6.42; urosome = 6.70). After a starved copepod resumed feeding for a few hours, the metasome region pH decreased from 6.33 (average) to 5.80, indicating the secretion of acidic digestive fluid. For *C. hyperboreus* that had been feeding on *T. weissflogii*, the urosome region was even more acidic (pH = 5.40–6.14).

Oxygen profiles—Five individuals of *Calanus hyperboreus* were successfully profiled for their gut oxygen content (Fig. 3). Oxygen undersaturation was recorded throughout the urosome region. When the gut was empty, oxygen level did not drop below 136 μmol L^{-1} (44.8%). When visible food remains were present in the urosome region, a stronger oxygen gradient was detected and it reached anoxia at 250 μm up the metasome region. Diet type also appeared to have an effect, because anoxia was reached within a shorter distance up the metasome region of the individual feeding on diatoms than the ones feeding on *Rhodomonas* (Fig. 3). This difference was complementary to the difference in gut pH between the same diets (Table 1).

The gut oxygen level was also measured in two individuals of *C. glacialis* that had been feeding on *R. salina* (Fig. 4). For the individual without visible food remains in the gut, dissolved oxygen concentration decreased from 304 μmol L^{-1} just outside the anal opening to 85.3 μmol L^{-1} (28% saturation) at the inner part of the urosome region, across a distance of ~ 600 μm, and oxygen continued to decrease to anoxia in the metasome region. As the tethered copepod continued to feed and generate food remains in the gut, oxygen level was further reduced to 230 μmol L^{-1} (75%) at the anal opening, and oxygen undersaturation even extended to 100 μm outside the anal opening (Fig. 4). Similar observations were obtained with a second, slightly larger, individual of *C. glacialis* with visible food remains in the gut: an oxygen gradient of 275.4–96.6 μmol L^{-1} (90.6–31.8%) was present in the urosome region, and the lower metasome region was strongly depleted in oxygen (Fig. 4). For *C. glacialis* it was not possible to push the sensor any deeper into the animal, but after cutting away the urosome, sensor measurements documented that the central metasome region was anoxic.

Discussion

Copepod gut pH and oxygen—We used the microelectrode technology to produce, to our knowledge, the first detailed pH and oxygen profiles of the gut of two *Calanus* copepod species from arctic and subarctic waters. Our results unambiguously show that parts of the animals' guts were acidic and undersaturated with oxygen relative to

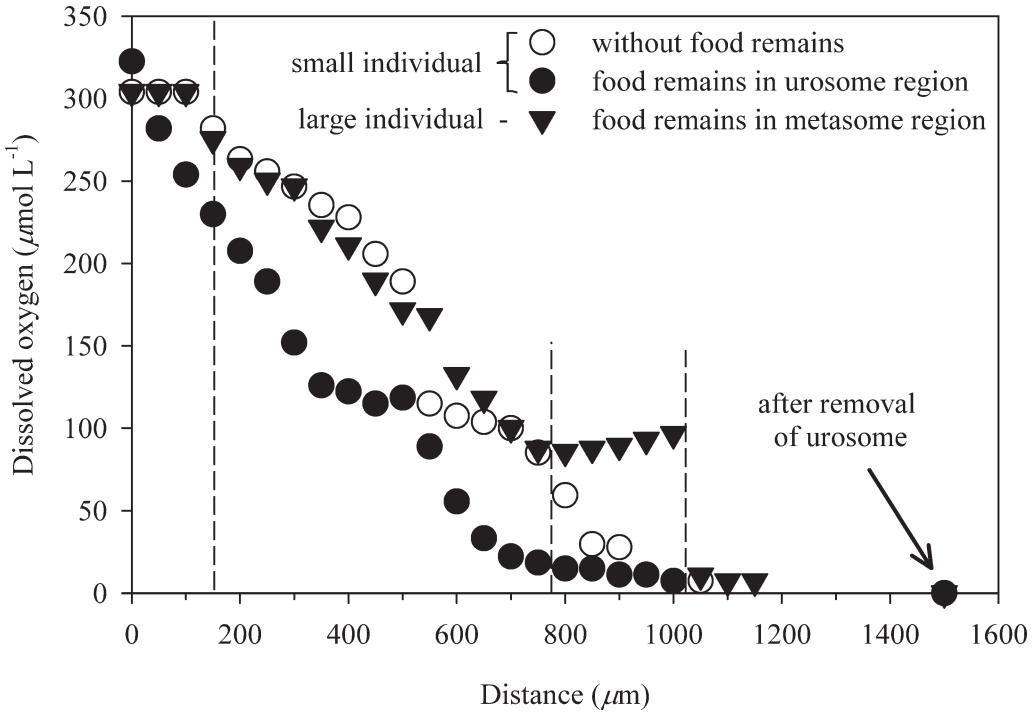

Fig. 4. Gut oxygen profiles of *Calanus glacialis* ($n = 2$) that had been feeding on *Rhodomonas salina*, without or with visible food remains in the gut. Distance was measured from ambient water into the gut. The left dashed line indicates the position of the anal opening. The middle and right dashed lines indicate the approximate positions of urosome–metasome transition for the small and large individual, respectively. After removal of urosome the sensor was inserted deeper into the metasome region and recorded anoxia in both individuals.

seawater. Using microelectrodes we were able to show that pH varied even within a short distance, and the pH range we observed in the gut (2.55) was also larger than what Pond et al. (1995) obtained with a pH-sensitive dye (1.73). In contrast to Pond et al. (1995), who showed that the gut of *C. helgolandicus* turned basic after feeding, we observed that the gut pH of starved *C. hyperboreus* decreased after feeding resumed, which is more consistent with the reported optimal pHs for digestive enzymes from *Calanus* copepod (Bond 1934).

In our experiments, a diatom diet induced a more acidic gut pH than a crytophyte diet, showing that diet composition can affect copepod gut pH. Literature data suggest that diatoms are more difficult to digest than flagellated microalgae (Conover 1966 as recalculated by Paffenhöfer and Köster 2005; Thor and Wendt 2010). One can speculate that the copepod gut produced more acidic fluid to aid the digestion of diatoms, although this is not necessarily related to the siliceous frustules because silica dissolution tends to occur faster in alkaline, not acidic, pH (Lewin 1961; Schlüter and Rickert 1998), and biogenic silica is often used as inert tracer in copepod feeding studies (Tande and Slagstad 1985; Cowie and Hedges 1996).

Diatoms can cause lower assimilation efficiency and higher fecal pellet production in copepods (Besiktepe and Dam 2002), and may, therefore, not only stimulate the copepod to secrete more digestive fluid (hence, lower gut pH), but also generate more organic and inorganic remains in the gut than a *Rhodomonas* diet. These rich food remains would then be subject to microbial respiration, leading to a stronger oxygen gradient as we observed with the diatom treatment relative to the cryptophyte treatment.

The oxygen profiles of *C. hyperboreus* and *C. glacialis* together indicate that microbial respiration inside the gut, especially when food remains (fecal pellets) were present, kept the gut environment suboxic to anoxic. Considering the high instantaneous gut evacuation rates of these copepod species (0.015 min^{-1} for *C. hyperboreus*, 0.017 min^{-1} for *C. glacialis*; Hansen et al. 1990), food would pass through the gut in only about an hour, implying that microbial activity must be very high in the gut to maintain the low oxygen level there. Complete anoxia was, however, detected only in the metasome region of both copepod species, even when food remains were present in the urosome region. This suggests that microbial respiration in the urosome region was not strong enough to completely deplete the oxygen supplied from the outside, and strict anaerobes may be more likely to thrive in the metasome region. On the other hand, it is also remarkable that microbial respiration in the gut could maintain oxygen undersaturation even outside the anal opening in one case.

Our measurements were confined to the urosome and lower metasome regions, and the pH and oxygen conditions of the cephalosome and upper metasome regions remained unknown. Pond et al. (1995) reported close to or slightly above neutral pH in the 'foregut' of *C. helgolandicus*, which appears to be equivalent to the cephalosome region as defined in our study. In another study, the upper half of a copepod's gut appeared blue after the copepod was exposed to a blue dye (Aniline Blue), indicating that

the copepod was drinking ambient water (Bickel et al. 2009). One may, therefore, expect to see a gradient decreasing from the ambient oxygen level at the buccal cavity toward anoxia in the metasome region of the gut.

Ecological and biogeochemical implications—The repeated observations of anoxia in the metasome region reaffirm the notion that copepod guts are microhabitats for strict anaerobes in an otherwise less habitable, well-oxygenated water column (Proctor 1997; Braun et al. 1999). The current measurements were made at fully air-saturated water. For copepods that live in O_2-depleted water, the suboxic or anoxic condition inside the animals may be even more extensive. Our observations also lend support to the suggestion that methanogenesis inside zooplankton gut can contribute to mid-water–column methane oversaturation (DeAngelis and Lee 1994). For bacteria to thrive inside these microhabitats, they must survive digestion and the acidic condition. The observed lowest pH (5.40) in our study is well within the tolerable range for most bacteria (Cotter and Hill 2003; Nojoumi et al. 2008), including anaerobes (Lowe et al. 1993), and diverse, viable bacterial communities have been recovered from copepod guts (Delille and Razouls 1994; Hansen and Bech 1996). On the other hand, the organic-rich environment inside a copepod gut may even support higher bacterial growth than the ambient ocean (Tang 2005).

The environmental conditions inside the gut may also influence the fate of trace metals ingested by the grazers. One example is iron, which is an essential micronutrient for phytoplankton (Martin et al. 1991). As ingested materials pass through a copepod's gut, the acidic and low-oxygen condition may facilitate iron dissolution and remineralization to the more reactive and soluble Fe (II) form. This may explain the reportedly high (> 50%) percentage of ingested iron being released in dissolved form when the copepod *Acartia tonsa* feeds on the diatom *Thalassiosira pseudonana* (Hutchins et al. 1995), and the process may be important for recycling this essential element within the pelagic zone and affecting the primary production in iron-limited regions (Hutchins and Bruland 1994).

The size of the microelectrodes limited our study to large copepod species that dominate subarctic and arctic waters. The dominant copepod species in tropical and subtropical waters are usually too small for microelectrodes. The gut oxygen profile of these warm-water species is of particular interest: on the one hand, the higher water temperature is expected to lower oxygen solubility and promote anoxia in the copepod's gut; on the other hand, the shorter length of the gut may lessen oxygen depletion. Future technological improvements, for example, by coating ingestible nano particles with oxygen- or pH-sensitive chemicals, may allow us to image the chemical conditions in the gut of these warm water species to evaluate the global significance of our findings.

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