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Endosymbionts escape dead hydrothermal vent tubeworms to enrich the free-living population

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Theory predicts that horizontal acquisition of symbionts by plants and animals must be coupled to release and limited dispersal of symbionts for intergenerational persistence of mutualisms. For deep-sea hydrothermal vent tubeworms (Vestimentifera, Siboglinidae), it has been demonstrated that a few symbiotic bacteria infect aposymbiotic host larvae and grow in a newly formed organ, the trophosome. However, whether viable symbionts can be released to augment environmental populations has been doubtful, because (i) the adult worms lack obvious openings and (ii) the vast majority of symbionts has been regarded as terminally differentiated. Here we show experimentally that symbionts rapidly escape their hosts upon death and recruit to surfaces where they proliferate. Estimating symbiont release from our experiments taken together with well-known tubeworm density ranges, we suggest a few million to 1.5 billion symbionts seeding the environment upon death of a tubeworm clump. In situ observations show that such clumps have rapid turnover, suggesting that release of large numbers of symbionts may ensure effective dispersal to new sites followed by active larval colonization. Moreover, release of symbionts might enable adaptations that evolve within host individuals to spread within host populations and possibly to new environments.

symbiosis | mutualism stability | symbiont seeding | tubeworms | Vestimentifera

The evolution of cooperation between species (mutualism) represents a challenge for evolutionary theory (1–5). The host provides benefits to the symbiont by supporting its partner's offspring at the expenses of its own offspring (6, 7). To assure beneficial coexistence after establishment of an association, partner sanctions and/or partner fidelity feedback may act as postinfection mechanisms (1, 8–12). During vertical transmission, hosts transmit symbionts directly to offspring during reproduction (1, 5, 7, 13). During horizontal transmission, partners must reassociate anew each host generation (13). Limited dispersal following release of the cooperating symbiont back into the environment might keep the offspring of both partners in close proximity (14–18) and thereby enhance the probability that the offspring of both partners can reassociate (7, 14, 16, 18–20). Therefore, the processes of symbiont release back into the environment are key factors in understanding interspecies cooperation in ecological and evolutionary timescales.

In facultative horizontally transmitted pathogens, escape from the infected host and long survival in the environment are crucial to reinfect susceptible hosts, especially in cases when host density is low (21, 22). Although some pathogens are capable of growing outside the host, others follow a sit-and-wait strategy in the absence of proliferation in the environment (21). Several studies have shown that host-associated and free-living beneficial symbiont populations apparently can rejoin. The majority of horizontally transmitted bioluminescent *Vibrio fischeri* housed in the light organ of the bobtail squid are expelled into the environment daily and remain viable for at least 1 d (23, 24). Arbuscular mycorrhizal fungi reproduce when associated with the plant host

and repopulate the soil to infect new vascular plants (25). Likewise, a dense population of nitrogen-fixing rhizobia reenters the soil upon root nodule senescence in legume hosts and remains there for years (25–27). Genetically identical symbiont lineages to those being released were shown to reinfect the next host generation in the squid and legume symbiosis (28, 29).

Transmission is also horizontal in the mutualism between the sessile tubeworm *Riftia pachyptila* and its thiotrophic gamma-proteobacterial endosymbiont *Candidatus Endoriftia persephone* (short Endoriftia) from deep-sea hydrothermal vents (30). Endoriftia exhibits an identical 16S rRNA sequence to the co-occurring hydrothermal vent vestimentiferan symbionts of *Tevnia jerichonana* and nearly identical 16S rRNA sequences to the symbionts of *Oasisia alvinae* and *Ridgeia piscesae* (31–33). A high level of genetic homogeneity in the internal transcribed spacer (ITS), with one dominant 16S rRNA phylogroup and ITS subtypes, is present in intracellular Endoriftia (34). While associated, *Riftia* provides all substrates necessary for chemosynthesis to the symbiont (35), which ultimately allows the symbiont to grow to more than a billion symbiont cells·g⁻¹ trophosome (36, 37). In return, the symbiont provides benefits through release of fixed organic carbon to the host, thus entirely nourishing the gutless host (38, 39). The intracellular symbionts are located in specialized host cells, the bacteriocytes, deep within the host body with no openings toward the exterior (40–42). The bacteria divide within unipotent bacteriocyte stem cells mostly as rods, convert into small and large cocci in the proliferating bacteriocytes, and are digested before host bacteriocyte apoptosis (43). Whereas in some mutualisms, release of symbionts may happen during the host's life, such as in the *V. fischeri*-squid (23, 24) and rhizobia-legume

Significance

For horizontally transmitted, facultative symbionts, cycles of infection and escape from the host are crucial for the persistence over host generations. The hydrothermal vent tubeworm *Riftia pachyptila* is entirely nourished by its thiotrophic endosymbiotic bacteria, which are acquired horizontally in settled larvae; however, release back into the environment has not been demonstrated. We show experimentally that viable symbionts are released upon host death. Moreover, observations of turnover of tubeworm clumps after a volcanic eruption provide evidence for rapid colonization, growth, and death. The observed connectivity of host-associated and free-living symbiont populations helps to explain the stability of this mutualism over ecological and evolutionary timescales.

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compared with vent conditions. In the deep-sea treatment the oxygen concentration was reduced from $86.2 \pm 0.8\%$ (0.5 d) to $10.0 \pm 0.2\%$ (from 6 d on) due to closed chambers. Whereas under the simulated deep-sea treatment the peak release occurred after 6 d, under vent conditions most symbionts were released in the half-day incubations (Table 1). These results indicate that the time window for symbiont escape may be relatively short under vent conditions but much longer under deep-sea conditions.

Symbiont Morphological Variability and Proliferation Activity. Because symbionts are differentiated into rods and cocci in the trophosome (42), we tested whether any of these morphotypes is preferentially released using FISH by simultaneously applying the symbiont-specific probe and the bacteria-specific probe mix. Symbionts recruited onto coverslips were composed of up to 3.3% rods (1.0–2.4 μm length, 0.5–0.9 μm width) and 96.7–100.0% cocci (0.5–13.6 μm diameter) (Fig. 24), indicating a morphological variability similar to the endosymbiotic population (2.1% rods, 97.9% cocci) (42). No significant differences in relative densities of morphotypes were detected in the different treatments and after different incubation times (Table 1). Some bacterial contaminations on the coverslips could be detected in some experiments, but did not statistically influence the colonization of symbionts. In contrast, the specific 16S rRNA phylotype of *Endoriftia* detected on glass slides deployed after 1 y in deep-sea vents in *Riftia* aggregations as well as on bare basalt under ambient deep-sea conditions was all rod-shaped (34). Nevertheless, the ITS of free-living *Endoriftia* might be variable, as it is within the host (34).

To analyze the viability and proliferation activity of released symbionts, we estimated the frequency of dividing cells (FDC) using DAPI and FISH staining simultaneously. An FDC of $4.9 \pm 0.8\%$ was detected on coverslips, whereas division could not be unambiguously determined in the symbiont populations detected in the water samples from the SRPs (Fig. S2). This further supports that escape and colonization are active processes of viable symbionts.

In contrast to restricted proliferation of rods and small cocci in the trophosome (43), all morphotypes of the released symbionts apparently divided after release and settling on surfaces (Fig. 2B), and the majority of proliferating symbionts were large cocci. These findings suggest that tubeworms control proliferation of *Endoriftia* during its symbiotic life, whereas *Endoriftia* released from host control initiates proliferation, similar to rhizobia in indeterminate root nodules of legumes (55, 56), which dedifferentiate

upon entering the soil (57). No statistically significant differences in FDC between vent and deep-sea conditions or an increase of FDC over time were found (Table 1). This pattern may be explained by a balance between proliferation and death in the recruited population that thus retained constant density over the experimental time frame. Similar behavior was found in rhizobia entering a viable but inactive stage upon release from legumes into the soil (25).

Estimates of Symbiont Escape and Colonization. Our experiments on trophosome tissue are indicative of processes that may happen when hydrothermal flux wanes. Due to the lack of substrate for chemosynthesis, the thiotrophic symbiont ceases to nourish the host, and ultimately the starving host dies. *Endoriftia* also must escape through the skin to leave the dead host. This migration process through the skin, albeit in opposite direction, has been observed during transmission (30). At the East Pacific Rise (EPR) 9°50'N region, tubeworm clumps were reported ranging from a few individuals only to large clumps with 2,000 individuals m^{-2} (58, 59). A medium-sized worm of 20 g wet weight with a 3-g trophosome (60, 61) houses about 1.11×10^{10} symbionts (36, 37). Under cold deep-sea conditions about 7×10^5 symbionts would escape from such a dead worm within half a day. Because *Endoriftia* must also escape through the skin to leave the dead host, further loss may be involved; however, this factor is currently difficult to quantify, so the estimates provided represent an upper bound. Taking the variability of tubeworm density in such patches into account, a remarkable seeding event of *Endoriftia* into the environmental population must occur when a tubeworm clump dies within a short time upon cessation of vent flux. Roughly between 7 million (7×10^6 estimated for 10 worms) and 1.5 billion symbionts (1.4×10^9 estimated for 2,000 worms) may enter the environment upon such an event. In situ experiments have shown that crabs rapidly feed on dead trophosome pieces (62). Although this fast scavenging behavior reduces the number of symbionts, it may at the same time facilitate escape of the remaining symbionts due to the crabs opening the body and/or sloppy feeding.

Monitoring of Tubeworm Clump Longevity at the EPR. *Riftia* is one of the fastest-growing invertebrates known (63); however, it may not live very long. We monitored several discrete tubeworm clumps from the sites Tica, P-Vent, and Sketchy at the EPR 9°50'N region in the area that was covered with new lava during the eruption in late 2005/early 2006 (64) between approximately

Table 1. Symbionts colonizing coverslips in high-pressure vessels under simulated, deep-sea, and flowthrough vent conditions

Incubation, d	Replicates	Symbionts, mm^{-2}	FDC, %	Rods, %	Length, μm	Width, μm	Cocci, %	Diameter, μm
Deep-sea								
0.5	3 (3,3,4)	16.3 ± 15.7	1.3 ± 2.0	0.0	N/A	N/A	100.0	4.2 ± 0.7
1	3 (3,3,5)	47.1 ± 60.0	3.3 ± 3.9	0.5 ± 1.2	2.3 ± 1.3	0.9 ± 0.5	99.5 ± 1.2	3.7 ± 0.5
2	3 (3,2,5)	30.4 ± 30.0	2.0 ± 3.5	2.8 ± 5.3	1.8 ± 0.9	0.9 ± 0.3	97.2 ± 5.3	4.3 ± 0.6
6	2 (3,5)	377.9 ± 507.5	20.0 ± 28.3	0.0	N/A	N/A	100.0	3.3 ± 1.2
8	1 (5)	9.8 ± 7.1	4.0 ± 6.9	0.0	N/A	N/A	100.0	4.2 ± 0.5
10	1 (5)	25.9 ± 29.0	1.6 ± 1.3	0.5 ± 1.1	1.6 ± 0.8	0.9 ± 0.5	99.5 ± 1.1	4.3 ± 0.6
Vent								
0.5	3 (3,3,3)	80.0 ± 133.8	7.4 ± 12.9	0.1 ± 0.4	1.5 ± 0.4	0.8 ± 0.2	99.9 ± 0.4	3.1 ± 1.4
1	3 (3,3,3)	51.1 ± 84.1	5.8 ± 8.2	0.0	N/A	N/A	100.0	2.5 ± 0.1
2	1 (3)	26.1 ± 42.9	4.3 ± 7.5	0.0	N/A	N/A	100.0	4.0 ± 0.2
6	2 (3,3)	2.1 ± 0.8	0.0	0.0	N/A	N/A	100.0	2.4 ± 0.1

Trophosome pieces were incubated under simulated deep-sea conditions at 4 °C and $171.6 \pm 0.5 \mu\text{mol L}^{-1}$ oxygen without flow or under simulated flow-through vent conditions at 22.4 ± 0.6 °C, $280 \pm 48 \mu\text{mol L}^{-1} \sum \text{H}_2\text{S}$ and $107 \pm 29 \mu\text{mol L}^{-1}$ oxygen. Density of symbionts released and settled on coverslips (FISH-positive cells stained with symbiont-specific probe, EUB338 probe mix, and DAPI were counted on 1.125 mm^2 of glass coverslips), frequency of dividing cells, relative density of rods (length and width), and cocci (diameter) are given as mean and SD. To analyze the morphological variability, a total of 100 rods and cocci (when present) was counted for each coverslip and length and width for rods and diameter for cocci were measured. Incubations were conducted in triplicate (three different specimens incubated in different high-pressure incubation vessels) for each treatment for 0.5, 1, and 2 d. The longer 6-d incubations under vent and deep-sea conditions were done in duplicate. In addition, simulated deep-sea incubations were also run for 8 and 10 d once. Replicates are given: The numbers refer to trophosome samples of different specimens, and the numbers in parentheses refer to the number of coverslips analyzed in total. N/A, data not available.

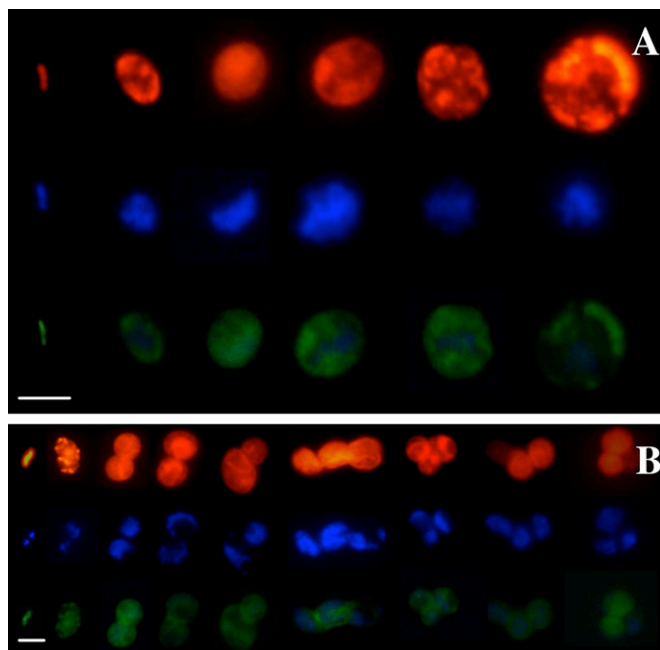


Fig. 2. Symbiont settlement on coverslips. (A) Symbiont population settlement on SRP glass coverslips: rod-shaped symbionts, small cocci, and large cocci of *Endorhiza* detected by FISH. (B) Symbiont settlement on SRP glass coverslips and division of settled symbiont morphotypes and formation of small colonies of the settled symbiont as detected by FISH along with symbiont DNA stained with the general nucleic acid stain DAPI. Characteristic samples are shown. Red, EUB338 probe mix targeting most bacteria; green, symbiont-specific probe RfTO445; blue, DAPI. (Scale bars, 5 μm .)

11 mo and 4 y posteruption (Table S3). The assessment of viability was done by in situ observations, videos, and collections. Clumps composed of more than 95% empty tubes were classified as dead. Initially, all patches were dominated by *T. jerichonana* [but also housing Endoriftia (31)] (Fig. 3 A–E). Although all patches at Sketchy were, with at most 2 y, relatively short-lived (Fig. 3 I), some patches at P-Vent and Tica were colonized longer than 1 y (Fig. 3 F–H) but less than 4 y (Fig. 3 J and K) or more than 4 y (Fig. 3 L), respectively. Initially, *Tevnia* dominated these vents, but within 2 y posteruption *Riftia* replaced the pioneer *Tevnia*, confirming an earlier study of the previous eruption in 1992 (58). This indicates that over a relatively short time, a tremendous amount of Endoriftia may escape dead tubeworms and increase the density of the free-living population.

Host-Symbiont Life-History Traits. Adult tubeworms are sessile, but dispersal is through aposymbiotic larvae in the pelagial (65) and facilitates recruitment to already-existing and newly opened vents. Likewise, the free-living symbiont must colonize such new vents. Hence, both partners must reach new vent sites separately, yet although larvae are motile, for symbionts this must be a passive process. The potentially pulsed release of symbionts from dying tubeworm clumps may ensure reliable colonization of nearby new vent fields. However, how new sites that arise often hundreds of kilometers away are colonized remains an open question but possibly involves a more directed dispersal process, either by currents or by carriage on motile animals that actively seek out new vent sites. In contrast to the highly disturbed vent environment, which requires dispersal of tubeworm larvae and symbionts to new sites, the dispersal of hatched juvenile squids as well as daily expelled *V. fischeri* (23, 24, 66) in the relatively stable subtidal sands may indeed be rather limited and in accordance with the benign nature of the habitat in which they thrive.

Conclusions

Our unique finding of Endoriftia escaping dead tubeworms and seeding the environment through a temporally and spatially highly dynamic process in this fluctuating and disturbed vent ecosystem confirms the connectivity from the host-associated population to the environmental population. Considering the maximal number of 20 symbionts, which was previously found to infect each host larva (30), and our estimates of 7×10^5 symbionts leaving a medium-sized worm upon host death may indicate the fitness benefits for Endoriftia to engage in a temporary association with tubeworms. The hosts provide nutrients for carbon fixation and growth to the symbiont in a competition-free habitat (67). The release of symbionts is in accordance with theoretical predictions and suggests a mechanism for adaptation that arises in individual tubeworms to spread among host populations. However, whether the symbionts infecting new hosts are indeed those that have been released from other dead tubeworms remains to be shown. Now that this principal mode of host-associated and free-living symbiont connectivity is known, we can start to decipher the stability of this mutualism in situ in the framework of population genetics and metapopulation ecology.

Materials and Methods

Monitoring Discrete Tubeworm Clumps in Situ. During four cruises with the research vessel (RV) *Atlantis* and the human-occupied vehicle (HOV) *Alvin* in October/November 2006, November/December 2006, November 2007, and December 2009, colonization devices were deployed in one patch at the sites Sketchy and P-vent each and in two patches at Tica (Table S3). The colonization of the foundation species *Riftia* and *Tevnia* and their health were monitored with live observations, videos, and collections of samples. Clumps were classified as dead when more than 95% of tubes were empty. In addition,

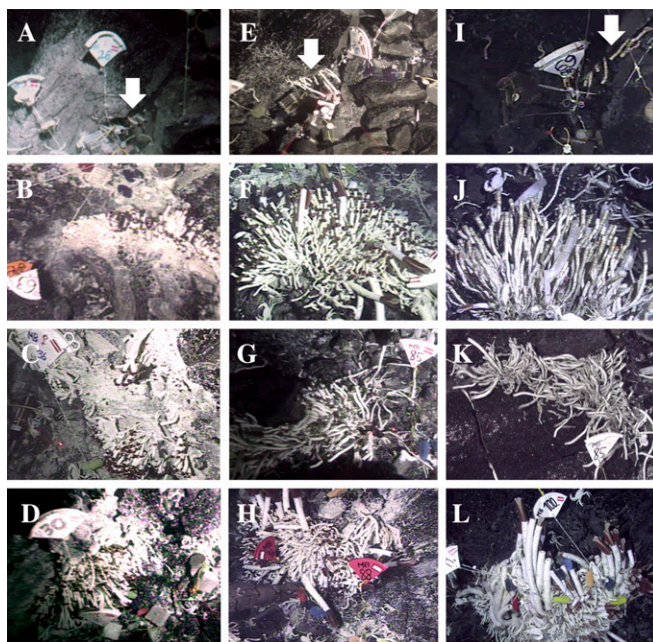


Fig. 3. Temporal colonization of vent patches over 4 y after a volcanic eruption in 2005/2006. Patches of *T. jerichonana* at Sketchy (A), P-Vent (B), Tica location 1 (C), and Tica location 2 (D) 11 mo posteruption. Same patches with live tubeworms (mostly *Tevnia*, but also *Riftia*) at Sketchy 1 y posteruption (E) and at P-Vent (F), Tica location 1 (G), and Tica location 2 (H) 2 y posteruption. Same patches with mostly dead tubeworms, assessed by their empty tubes, at Sketchy (I), P-Vent (J), and Tica location 1 (K) 4 y posteruption. Patch of live *Riftia* and *Tevnia* at Tica location 2 (L) 4 y posteruption. Note one live *Riftia* among empty tubes in J, Lower Left. Note that the two laser points in E and G mark 10 cm; figures show a slice of a bucket lid for size comparison. Arrows highlight tubeworms at Sketchy in A, E, and I.

upon recovery of the devices and samples, the viability of small tubeworms was checked using a dissection microscope. Temperature, a proxy for vent flow (48), was measured in situ with the low-temperature probe of *Alvin*.

Collections of Vestimentiferans and High-Pressure Incubations. Vestimentiferan tubeworms were collected by the R/V *L'Atalante* with the deep-sea submergence vehicle (DSV) *Nautille* in May 2010 and by the R/V *Atlantis* and remotely-operated vehicle (ROV) *Jason* in November 2011 at the hydrothermal vent sites Tica and north of P-Vent at the East Pacific Rise. Tubeworms were dissected and incubated aboard the ship. To follow the symbiont escape process, we used custom-designed symbiont recruitment plates equipped with glass coverslips in high-pressure vessels to simulate deep-sea conditions of bottom water with $175 \mu\text{mol}\cdot\text{L}^{-1}$ oxygen (65) and about 2–3 °C at the basalt surfaces in the axial summit trough of the EPR (68). Pressure vessels for the simulated deep-sea treatments were filled with 0.2 μm sterile-filtered seawater and kept at 4 °C without flow and $171.6 \pm 0.5 \mu\text{mol}\cdot\text{L}^{-1}$ oxygen at the beginning (short deep-sea conditions; *SI Materials and Methods*). To approximate vent habitat conditions for thriving *Riftia* (46–48) and previous maintenance conditions (49), simulated vent conditions (short vent conditions) were performed with continuous flow at 22.4 ± 0.6 °C, $280 \pm 48 \mu\text{mol}\cdot\text{L}^{-1} \sum \text{H}_2\text{S}$ [i.e., sum of all forms of dissolved sulfide; short sulfide (46)] and $107 \pm 29 \mu\text{mol}\cdot\text{L}^{-1}$ oxygen for up to 6 d. Approximately 0.4 g wet weight freshly dissected trophosome was filled but not squeezed into micro-porous specimen capsules (pore size: 120–200 μm) and incubated in triplicate (three specimens in one vessel each) for each treatment of deep-sea and vent conditions for 0.5, 1, and 2 d, each. The longer 6-d incubations under vent and deep-sea conditions were done in duplicate. In addition, deep-sea incubations were also run for 8 and 10 d once. As controls, trophosome pieces were fixed in 100% ethanol or frozen in liquid nitrogen to kill symbionts before incubation under deep-sea conditions for 0.5 and 1 d once (*SI Materials and Methods*). During experiments, the sulfide concentration (47), salinity, oxygen, and temperature were monitored continuously.

Specimen Fixation and Preparation. At the end of each experiment, the entire water (28 mL) of the pressure vessels for SRP incubations was filtered (Millipore; GTPP 0.22 μm). Incubated trophosome, glass coverslips, and filters were fixed for fluorescence in situ hybridization in 100% ethanol or 4% (wt/vol) paraformaldehyde, and trophosome was embedded in LR White resin [London Resin (30)]. One-micrometer semithin sections were cut using a Leica EM UC7 microtome. For transmission electron microscopy, incubated trophosome was fixed in 5% (wt/vol) glutaraldehyde, 4% (vol/vol) formaldehyde, embedded in low-viscosity resin (Agar Scientific), and cut into 70-nm ultrathin sections (*SI Materials and Methods*).

Fluorescence in Situ Hybridization and Transmission Electron Microscopy. FISH was performed as previously described (30). As a positive control, glass coverslips and filter pieces were hybridized simultaneously with the probe RifTO445 (short symbiont-specific probe) (30) and the general bacterial probe mixes EUB338 I, II, and III (Table S1). The nonsense probe NON-388 EUB was used with the same fluorescence label as the probes on a separate coverslip or on a filter piece of the same treatment as a negative control. DAPI (4',6-diamidino-2-phenylindole) was used as a general DNA counterstain. Simultaneous hybridizations of host probe, symbiont-specific probe, and general bacterial probe mix were carried out at 35% (vol/vol) formamide concentration.

All filters and glass coverslips were analyzed with the *Riftia* host probe RP1752 (Table S1) with a Zeiss Axio Imager epifluorescence microscope. Additionally, filtered water sampled before the experiments was checked by FISH and 16S rRNA gene PCR (*SI Materials and Methods*) using the symbiont-specific probe RifTO445 and the primers RifTO44 and RifTO445, respectively (30, 34). For TEM investigations, 30 randomly selected pictures were taken from the central and median lobule zones (43) of each trophosome with a Zeiss EM 902 transmission electron microscope. The average of symbiont counts for each replicate and each treatment, as well as the percentages compared with the freshly fixed trophosome under initial conditions taken as 100%, is listed in Table S2 (Fig. S3).

Counting and Statistical Analyses. Whole-glass coverslips and filters were investigated for symbiont density and proliferation (estimated by the frequency of dividing cells). All symbiont cells stained simultaneously by the symbiont-specific probe, the EUB338 probe mix, and DAPI were counted on 1.125 mm² of three glass coverslips each and on 0.156 mm² of one filter, containing all the water from the incubation vessel. Bacteria other than the symbiont (positive with EUB338 probe mix, negative with symbiont-specific probe) were also counted. To test whether the bacteria had an influence on the recruitment behavior of Endoriftia, we used the nonparametric Kruskal–Wallis test ($P < 0.01$). To test for differences between treatments and time in density of FDC of symbionts on coverslips and in the water and of symbiont decay in numbers in fresh and incubated trophosome (Fig. S3 and Table S2), we used the Kruskal–Wallis test ($P < 0.01$) for several independent samples to compare the means of more than one dataset. To compare each of the treatments, time of incubation, and symbiont decay in number, we used the multiple comparison post hoc Tamhane test for unequal variances ($P < 0.01$) in SPSS 22.0 (IBM).

Symbiont Release/Recruitment Model. To estimate how many symbionts may escape after host death and may colonize surfaces in nature, we took the mean density of symbionts we found after half a day under deep-sea conditions on a coverslip and extrapolated this density to the entire inner surface of the incubation vessel (5772.7 mm²) and related the 0.4 g incubated trophosome to the mean symbiont density ($3.7 \times 10^9 \text{ g}^{-1}$ trophosome) (36) to the mean trophosome weight ($15.3 \pm 4.9\%$ of total host's wet weight) (60) in a tubeworm with 20 g wet weight (35). To estimate the impact of symbiont release after the waning of vent flux, we extrapolated the minimal release from one worm to the ranges of tubeworm clumps (11 individuals in a small clump covering 0.02 m² basalt to 2,000 individuals·m⁻² in a large clump) (58, 59) reported from this vent region.

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