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Phytophthora ramorum and *Phytophthora gonapodyides* Differently Colonize and Contribute to the Decomposition of Green and Senesced *Umbellularia californica* Leaves in a Simulated Stream Environment

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Abstract: Plant pathogenic as well as saprotrophic *Phytophthora* species are now known to inhabit forest streams and other surface waters. How they survive and function in aquatic ecosystems, however, remains largely uninvestigated. *Phytophthora ramorum*, an invasive pathogen in California forests, regularly occurs in forest streams, where it can colonize green leaves shed in the stream but is quickly and largely succeeded by saprotrophically competent clade 6 *Phytophthora* species, such as *Phytophthora gonapodyides*. We investigated, using controlled environment experiments, whether leaf litter quality, based on senescence, affects how *P. ramorum* and *P. gonapodyides* compete in leaf colonization and to what extent each species can contribute to leaf decomposition. We found that both *Phytophthora* species effectively colonized and persisted on green or yellow (senescing) bay leaves, but only *P. gonapodyides* could also colonize and persist on brown (fully senesced and dried) leaves. Both *Phytophthora* species similarly accelerated the decomposition of green leaves and yellow leaves compared with non-inoculated controls, but colonization of brown leaves by *P. gonapodyides* did not affect their decomposition rate.

Keywords: leaf decay; oomycetes; invasive species; aquatic fungi; trophic specialization; saprotroph; pathogen; parasite

1. Introduction

The ecology of *Phytophthora*, a genus of fungal-like oomycetes historically erected and known for plant pathogenic species primarily associated with destructive diseases in agriculture [1], has undergone substantial reconsideration in recent years [2]. The recent emergence of a number of *Phytophthora*-caused plant epidemics in forests and other non-agricultural ecosystems has clearly shown that many members of the genus have potential as invasive species that can threaten natural ecosystems [2–4]. As a consequence of research in non-agricultural environments, a surprising diversity and abundance of *Phytophthora* species have been discovered, many previously undescribed [2,4]. Incidental to this research has been the discovery that many species of *Phytophthora* are abundant in natural surface waters, especially in streams. Many such species are so widespread and regularly encountered that they are now considered resident, if not endemic, and characteristic of such environments [5–22]. Nevertheless, isolates of well-known plant pathogenic species or species complexes are also regularly recovered, often without discernible symptoms or signs of disease on the vegetation [7,9,15,16,21,23–26].

Though the prevalence of *Phytophthora* in surface waters is now well established, the ecology underpinning this phenomenon is largely speculative. Because these organisms are known primarily

as causes of often devastating plant diseases, the nature of their presence in these environments and its implications for the persistence and spread of pathogenic species are important considerations for disease prevention and management. There is also a growing interest to understand the role of *Phytophthora*, among other Peronosporales, in decomposition of vegetative matter in aquatic environments [27]. The biology of *Phytophthora*, a genus of well adapted plant pathogens with a necrotrophic phase [1,28], suggests that their ecological role in leaf decomposition should be early colonization and breakdown of relatively fresh, live vegetative tissue. As they colonize leaves newly exposed in streams, they can open the integral tissues for colonization by saprotrophic organisms less able to penetrate the leaf cuticle, in a process analogous to 'conditioning' of leaf litter for palatability to shredder organisms [29,30]. The co-occurrence of both known plant pathogens and primarily stream-associated *Phytophthora* in aquatic environments also raises the question of whether these taxa have similar or divergent modes of life and whether they compete for resources in these environments.

In streams, vegetative litter is the primary source of nutrients for microorganisms [29,31,32], but the quality of vegetative tissues available varies with respect to senescence and degree of decomposition. Coastal forests of northern California largely consist of evergreen trees and shrubs [33] and so green leaves are a regular component of leaf litter introduced into streams, especially in winter and spring when, based on the region's climate, most rainstorms occur. Nevertheless, much vegetative litter is in the form of senesced leaves [34]. California bay (*Umbellularia californica* (Hook. and Arn.) Nutt.) is a common, broadleaf evergreen component of northern California's coastal forests and a frequently occurring tree species in riparian zones [35,36]. It is also a primary source of *P. ramorum* inoculum in California forests affected by sudden oak death, epidemic mortality of certain species in the beech family (Fagaceae) resulting from *P. ramorum* infection of the vascular cambium of the main trunk [37,38]. California bay leaves are highly conducive to sporulation by *P. ramorum* which, despite causing localized necrotic lesions and spots on leaves, nevertheless causes little damage to the tree species itself [39–41]. Additionally, bay leaves are sclerophyllous, as is typical for broadleaf evergreen plants in this Mediterranean climate, and so they decompose slowly [33]. Bay leaves are therefore both very common as leaf litter in northern California forest streams and a highly suitable substrate for *P. ramorum*.

Leaf senescence in California bay increases in the hot and dry summer months, peaking in late summer [34,37]. Thus, though green leaves often enter streams during winter and spring storms, as summer progresses, most of the bay leaves shed into streams are either dropped directly upon senescence from trees or are blown in from accumulated litter on the forest floor, nearby (as described by [29,42]). In general, fully senesced leaves have as much as 75% reduced protein content compared with green leaves, primarily from the dismantling of chloroplasts, and though yellow, senescing leaves still have live cells with active mitochondria, leaves that have turned brown as a result of drying no longer contain biologically active cells [30,43,44]. Therefore, green, senescing and fully senesced bay leaves are substrates that likely vary in their suitability for colonization by *P. ramorum* and stream-resident clade 6 *Phytophthora* species, taxa that commonly occur at high inoculum levels in northern California coastal forest streams [45,46].

We have shown that there is a difference in trophic specialization between the saprotrophically competent, clade 6 *Phytophthora* species, such as *P. gonapodyides*, and *P. ramorum* [45], an aggressive pathogen on many plant species [38,47,48]. In that study, green California bay leaves were rapidly colonized by *P. ramorum* in streams but were succeeded nearly completely within three weeks by clade 6 *Phytophthora* species [45]. It remains uncertain, however, whether *P. ramorum* was displaced by more competent saprotrophs or receded from an inability to persist in tissues that it had colonized as they progressively decomposed. Additionally, as most leaf litter consists of senesced leaves, it is important to know how these differently adapted taxa can compete for and persist on biologically inactive leaf tissue. Finally, though stream resident *Phytophthora* species are assumed to contribute to leaf decay given their regular recovery from streams and frequent association with decomposing vegetation [2,27], experimental evidence for the kind and extent of this contribution is lacking. Moreover, it is unknown

how the introduction of an exotic and plant pathogenic species, like *P. ramorum,* into a stream ecosystem might affect the decomposition of leaf litter by other organisms, such as resident *Phytophthora* species. Therefore we undertook a laboratory study to determine: (1) How well *P. ramorum* and *P. gonapodyides* could use senesced leaves as a substrate in comparison to green, live leaves, (2) whether colonization by and persistence of *P. ramorum* on leaves was affected by competition with *P. gonapodyides*, and (3) how much each of these *Phytophthora* species contribute to the decay of each leaf type.

2. Materials and Methods

2.1. Experiment Overview

To test the capacity of *P. ramorum* and *P. gonapodyides* to colonize green and senesced bay leaves, we conducted controlled environment experiments exposing leaves to an inoculum of each species alone and in combination in microcosms designed to simulate an aquatic environment (Supplemental Figure S1). The experiment consisted of a randomized complete block design with treatments representing a complete factorial of bay leaf type (green/live or brown/senesced), stream water addition (autoclaved or not), and *Phytophthora* inoculation (none, *P. ramorum*, *P. gonapodyides*, or combined *P. ramorum* and *P. gonapodyides*). These 16 treatment combinations were replicated in five blocks arranged in three growth chambers (model PGR-15, Conviron Controlled Environment Ltd). The experimental unit was a mesh packet of five leaves which were sampled at intervals over 16 weeks from microcosms. One treatment packet per sampling served for decomposition as percent biomass loss and another for colonization based on isolations on a selective medium. We repeated the experiment once, with leaf types maintained in the same microcosm in the first and in separate microcosms in the second experiment. We conducted a separate experiment with yellow, senescing leaves collected while still attached to trees and with the cuticle intact, with *P. ramorum*-only and combined *P. ramorum*/*P. gonapodyides* treatments as well as non-inoculated controls, in a completely randomized design with four reps in a single growth chamber.

2.2. Experiment Preparation

2.2.1. Leaves

We collected leaves from two sites where our previous field experiments were conducted [45]. One was a canyon through which Graham creek runs at Jack London State Park (38°21′2″ N, 122°33′16″ W) which consists of redwood forest with California bay as a dominant riparian tree, along with redwood (*Sequoia sempervirens* [Lamb. ex D. Don] Endl.), Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco), tanoak (*Notholithocarpus densiflorus* [Hook. & Arn.] P.S. Manos, C.H. Cannon, & S.H. Oh), bigleaf maple (*Acer macrophyllum* Pursh), and less frequently, madrone (*Arbutus menziesii* Pursh) [49,50]. The second included canyons around Copeland Creek at Sonoma State University's Fairfield Osborn Preserve (38°20′37″ N, 122°35′41″ W) which is characterized by mixed evergreen forest with a prevalence of California bay, white alder (*Alnus rhombifolia* Nutt.), big leaf maple, and occasionally, tanoak, madrone and coast live oak (*Quercus agrifolia* Née) [39,49]. At each site, we collected green, symptom-free bay leaves with a mature cuticle from trees and brown, recently shed bay leaves from beneath trees in the manner of Wood et al. [51]. Brown leaves were collected from both sites in September 2014, allowed to air dry in the laboratory, and then stored in sealed plastic bags at room temperature until used in experiments. Green leaves were collected on 12 December 2014 from the redwood forest site and on 5 August 2015 from the mixed evergreen forest site. Yellow leaves were collected directly from trees at the mixed evergreen forest site on 7 September 2015. Green leaves were stored at 4 °C for up to three weeks prior to use in experiments and yellow leaves were likewise stored but deployed in experiments within one week of collection. We collected leaves primarily from riparian areas around the described creeks, though, at the mixed evergreen forest site, we had to seek symptomless leaves to some extent from plateaus above the canyons. Leaves collected from each forest type were used in separate

experiments. Leaf treatments were in the same microcosm for the first experiment (40 containers) using leaves from the redwood forest and separate in the second experiment (80 containers) with leaves from the mixed evergreen forest. Yellow leaves were collected from the mixed evergreen forest site only. We tested a subsample of 50 of each leaf type for both sites—through isolations attempted on a selective medium as described below—to verify that there were no pre-existing *Phytophthora* infections. Brown leaves were soaked in sterile deionized water at 4 °C for two days prior to these test isolations. Leaf packets were prepared for each leaf type by packing five leaves into a flat envelope of 1 mm plastic mesh approximately 20 × 20 cm so that the leaf surfaces were in minimal contact with one another and each packet was sealed by folding over the open lip and securing it with two common metal staples.

2.2.2. Microcosms and Water

We assembled microcosms simulating an aquatic decomposition environment similar to the approach described by Medeiros et al. [52] (Supplemental Figure S1). White plastic buckets (2 gal., 21 × 24 cm, dia. × ht., Argee Corp, Santee, CA, USA) were used in the first (leaf types together) and yellow leaf experiments and opaque plastic containers (8 qt., 19.4 × 27.3 cm, dia. × ht., Continental Carlisle, Oklahoma City, OK, USA) in the second experiment (leaf types separate). Each container was aerated through a tube terminating in an aeration stone (3 cm dia., Uxcell®, Hong Kong, China) fed by an air pump (Commercial Air 1, EcoPlus®, 18W, 793 GPH, 12/Cs, Hawthorne Gardening Co, Vancouver, WA, USA) that was turned on for 30 minutes twice daily using an electric timer (Intermatic TIME-ALL®, TN311, Spring Grove, IL, USA). Aeration intensity was moderated with the addition of adjustable valves inserted in the tubing. A dilute nutrient solution was used as the base for the water mixtures in microcosms in order to avoid osmotic stress on spores. This was achieved by adding Hoagland's #2 salts (Caisson Laboratories, Inc., Smithfield, UT, USA) to autoclaved Millipore®filtered water for a final concentration of 0.01× the standard concentration (1.63 g/L). To test for any effect of natural stream microbiota on *Phytophthora* colonization or leaf decomposition, we included an addition of autoclaved or non-sterilized stream water as a treatment factor. The final composition of water in microcosms consisted of 4 L nutrient solution and 2 L stream water in the first experiment, and 4 L nutrient solution and 1 L stream water in the second. We collected water from streams in a bucket, pouring it through several layers of cotton mesh ("cheesecloth") into 4 L plastic bladders that we consolidated into larger plastic containers or used directly to transport water out of the field. Once brought to the laboratory, stream water was stored in plastic containers in a growth chamber at 12 °C and 12 h photoperiod (≈ 1800 lux) for 20 and 23 days prior to deployment in the first and second experiments, respectively. After storing the water for seven days, we submerged symptomless California bay leaves collected at each site as baits in each container for two days to confirm that *Phytophthora* zoospores were not present. We tested baits for infection using the isolation technique described below. No *Phytophthora* infections were detected from baits at this point. In the experiment with yellow leaves, we used only 4 L of a nutrient solution without stream water addition.

We measured stream pH, electrical conductivity (EC) and temperature on site at the time of stream water collection and subsequently in each microcosm throughout the experiments with a portable sensor (Combo pH and EC tester, model 98129N, Hanna Instruments, Woodsocket, RI, USA). Stream pH, EC, and water temperature were 8.55, 208 µS/cm, and 13.5 °C, respectively, for the redwood forest stream on the 9 December 2014 collection date, and 8.08, 363 µS/cm, and 17.7 °C, respectively, for the mixed evergreen forest stream on the 5 Aug 2015 collection date. To approximate natural stream pH in microcosms, we amended the mix of dilute nutrient solution and stream water in each microcosm with potassium carbonate buffer ("pH UP", General Hydroponics, Santa Rosa, CA, USA) at approximately 10 mg/L and adjusted it with KOH and HCl for a target of pH 8.3. The average pH (± SD) measured in microcosms periodically over the course of experiments was 7.99 (±0.27), 8.33 (±0.38) and 8.24 (±0.27) in the first, second and yellow leaf experiments, respectively. The average EC (±SD) was 208 (±63), 141 (±18), and 98 (±15) in the first, second, and yellow leaf experiments, respectively.

In the first experiment, where green and brown leaves were maintained together in treatment microcosms, the water darkened from leaf leachates shortly after experiment initiation. After 52 days, we removed two liters of water from each microcosm using an auto-siphon—sanitized with a 10% bleach solution in between each treatment—and added a fresh sterile nutrient solution to bring the volume back up to six liters. For the second experiment, we leached leaves prior to deployment in the experiment in approximately 300 mL autoclaved Millipore®-filtered water per 10 leaves and the water did not darken to the extent observed in the first experiment.

During all experiments, we periodically topped off the microcosms with autoclaved Millipore®-filtered water to 6, 5 or 4 L in the first, second, and yellow leaf experiments, respectively.

2.2.3. *Phytophthora* Inoculum

Phytophthora inoculum consisted of three isolates per species grown for three weeks at 20 °C in 10 mL 10% clarified V8 juice liquid culture (V8®original vegetable juice (Campbells Soup Co., Camden, NJ, USA) neutralized with 15 g/L $CaCO_3$, clarified by centrifuging at 7000 RPM for 10 minutes and diluted with deionized water) for the first experiment and in 5 mL of the same liquid culture for the second and yellow leaf experiments. Inoculum was introduced as mycelial mats to each container to initiate experiments with leaf packets already present for 24 h. Each container received six total inoculum doses: Those receiving only *P. ramorum* or *P. gonapodyides* receiving two doses of each isolate and the combined inoculation treatments receiving one dose of each isolate of each *Phytophthora* species. We used the same isolates in both experiments, all collected from the stream at the redwood forest site described above. *Phytophthora ramorum* isolates Pr-1906, Pr-1907, Pr-1908 and *P. gonapodyides* isolates P-1903, P-1904 and P-1905 are maintained in D.M. Rizzo's laboratory. Isolates of both *Phytophthora* species were originally identified by morphology, and the identity of *P. gonapodyides* isolates was confirmed through ITS sequence BLAST matches in GenBank (GenBank accessions: MK908979, MK908980, MK908981).

2.2.4. Experiment Conditions and Sampling

Experiments were maintained with 12 h photoperiod (≈1800 lux) and 18/14 °C light/dark temperatures, respectively, to reflect typical average stream temperatures and also to provide a temperature differential that would potentially encourage *Phytophthora* zoospore release. Temperatures were monitored hourly in each block using iButton®loggers (Maxim Integrated, Inc., San Jose, CA, USA) to verify chamber settings. At 4, 8 and 16 weeks after inoculating microcosms, we sampled one leaf packet for evaluating leaf decomposition as biomass loss and another for evaluating *Phytophthora* colonization. For experiments with green and brown leaves, we included an additional sampling for *Phytophthora* colonization at two weeks. Therefore, in the first experiment each microcosm contained seven packets of each leaf type for a total of 14, and, as leaves were maintained in separate microcosms in the second experiment, each contained a total of seven packets containing either green or brown leaves. In the yellow leaf experiment, microcosms contained six packets each.

2.3. Data Collection

To determine the rate of decomposition measured as leaf biomass loss [53,54], we weighed leaves to the hundredth decimal of a gram with an analytical balance (model EP612C, Ohaus Corporation, Pine Brook, NJ, USA) prior to packing and we labeled the packets with aluminum tree tags secured with a plastic tie for future identification. We estimated the original dry mass of both leaf types from the average dry weight (determined after oven-drying at 55–60 °C for 48 h) of a subsample of 50 fresh or air dried leaves. The average percent dry weight (±SD) for green and brown leaves, respectively, was 40.9 (±2.3) and 94.3 (±0.3) for the first experiment and 53.5 (±0.4) and 92.6 (±0.3) for the second experiment. The average percent dry weight for yellow leaves was 55.0 (±0.3). The average estimated weight in grams (±SD) for five green leaves was 0.84 (±0.08) and 1.13 (±0.06), and that for five brown leaves, 0.91 (±0.08) and 0.85 (±0.06) for the first and second experiments, respectively. For five yellow leaves, the

estimated average weight in grams was 0.98 (±0.06). At each sampling, leaves were retrieved from tagged leaf packets, rinsed gently with deionized tap water to remove adhering debris, oven-dried in a paper envelope or an open aluminum foil envelope at 55–60 °C for 48 h, and weighed as described above. The fraction of original biomass was calculated for all leaves in a packet by dividing the weight at the time of sampling by the estimated original dry biomass.

To determine the level of *Phytophthora* colonization of leaves, at each sampling we collected a packet for each leaf type from each container to evaluate by culturing on *Phytophthora*-selective PARP-H medium (corn meal agar 1.7% w/v, pimaricin 5 ppm, ampicillin 250 ppm, rifampicin 10 ppm, PCNB (pentachloronitrobenzene) 50 ppm and hymexazol 25 ppm, [1]). Upon retrieval, leaves were submerged and gently rubbed free of biofilm in 1% household bleach solution (≈65 ppm hypochlorite), surface sterilized in fresh bleach solution for three to seven minutes, rinsed with deionized tap water, and then laid out on paper towels and the excess water allowed to evaporate. Finally, leaves were wrapped in a paper towel and stored at 4 °C until isolations by culturing could be performed. Isolations were attempted from all leaves belonging to treatment (a single packet) using a 'mosaic' sampling approach whereby the leaf discs are removed from the petiole, midrib and flanking lobes of the leaf at approximately 1 cm distance from one another in order to collect a representative sample from the entire leaf [45,55]. For experiments with green and brown leaves, isolations were initiated immediately after collection, with most samples (75%) processed within 29 days. All isolations were completed by 46 days after collection. Storage period did not alter results when included as a covariate in models for these experiments and was excluded from the final analyses. Isolations from leaves of the yellow leaf experiment were completed within nine days after collection, and all isolations from a single collection week were completed in one day. The presence of *P. ramorum* and *P. gonapodyides* was determined by microscopic examination of isolate morphologies directly from the isolation plates after four to five days and checked again periodically for three weeks [45].

To test for active sporulation from colonized leaves in the microcosms, periodically a California bay leaf disc (12 mm dia.) was floated as bait—either naked or in a roughly 35 mm^2 mesh envelope—on the surface of the water in each microcosm for three to seven days, after which it was surface sterilized and isolations attempted from it on selective PARP-H medium. We conducted these tests of sporulation four times during the first and yellow leaf experiments, and three times during the second experiment. Additionally, we tested for sporulation periodically for up to eight weeks after all leaves had been removed from microcosms to determine if *Phytophthora* spores could persist in the absence of a substrate.

The first experiment was initiated on 29 December 2014, but we delayed the first collection at two weeks by two additional weeks because zoospores were not detected in the microcosms until two weeks after inoculation, most likely due to excessive aeration of the water during the first week. All subsequent collection dates were shifted forward by two weeks accordingly. Collections are reported according to the originally planned intervals of 2, 4, 8 and 16 weeks, with time zero being two weeks after inoculation. The final collection for the first experiment was on 1 May 2015 (126 days). Subsequent experiments proceeded as expected and the collection week reflects the period elapsed since introducing inoculum. The second experiment was initiated 28 August 2015 and concluded with the last sampling on 18 December 2015 (112 days). The yellow leaf experiment was initiated on 13 September 2015 and the final collection made on 7 January 2016 (116 days).

2.4. Analysis

Due to the differences in how each experiment was set up, we analyzed results separately for each.

2.4.1. *Phytophthora* Colonization

To evaluate the colonization of leaves by each *Phytophthora* species in each treatment, we recorded the total number of pieces yielding *P. ramorum* or *P. gonapodyides* out of the total number of pieces sampled for each leaf. The average proportion of leaves colonized by either species was calculated for each packet from this ratio. This average leaf fraction colonized per packet was *logit* transformed

to normalize variances, with a +0.005 correction applied to values of zero and −0.005 to values of one before transformation [56]. The transformed average proportions colonized were analyzed in linear mixed models (*lme* function) with the *nlme* package [57] in R statistical software, version 3.3.1 [58]. Replication block and microcosm were set as random variables with microcosm nested in a block. Because *Phytophthora* recovery followed a non-linear trend with respect to time, we treated the collection week as a categorical variable. As one *Phytophthora* species occurred almost exclusively in each treatment (see Results below)—*P. ramorum* and *P. gonapodyides* in the treatments where they were inoculated solely and *P. gonapodyides* in the combined inoculations—we simplified the analysis by comparing leaf colonization by the dominant species across treatments. That is, the response variable in the model was the average fraction of leaf discs colonized by *P. ramorum* in *P. ramorum*-only treatments, and by *P. gonapodyides* in *P. gonapodyides*-only and combined *Phytophthora* inoculum treatments. Therefore, the main independent variables for *Phytophthora* leaf colonization analyses were the inoculation treatment—with non-inoculated treatments excluded—and collection week. Leaf type (green or brown) and stream water type (autoclaved or not) were included as independent variables in the model for the experiments where the distinctions applied. The full set of interactions were included in the models for each experiment (see supplemental Tables S1−S4 and S6). We verified adherence to model assumptions by the Shapiro−Wilk and Levene's tests. We obtained *P*-values using the *anova* function in R with the sum of squares set to type III ("marginal"), and least square means comparisons with the *lsmeans* package [59]. Significance for means comparisons was determined with the default Tukey's HSD.

2.4.2. Leaf Decomposition

For leaf decomposition, we estimated a decay constant (k) for each treatment combination in each block based on the fraction of estimated original leaf mass remaining at each collection interval [53,54]. For this, we used the exponential decay equation $M_t = M_0 \cdot e^{-kt}$ where t is time as the number of incubation days, M_t is the fraction of leaf mass remaining at each collection interval, and M_0, fraction at time zero, is set to one [53,54]. Values for k were estimated using the *nls* function in R statistical program. The decay constants for each treatment combination were then analyzed in a mixed model using the *lme* function of the *nlme* package with inoculum, leaf and water type as independent variables and block as a random factor. For the yellow leaf experiment, only inoculum was used as an independent variable, and since replications were not blocked, an analysis of variance was performed using the *aov* function in R. For all experiments, we included treatments not inoculated with either *Phytophthora* species in the analysis to evaluate the effect of *Phytophthora* colonization on leaf decay. Two non-inoculated microcosms in the first experiment were contaminated with both *Phytophthora* species, and one non-inoculated microcosm in the second experiment became contaminated with *P. ramorum*, likely from a rare, undetected leaf infection. We excluded the results from these microcosms from the analysis.

3. Results

3.1. Phytophthora Leaf Colonization

When *P. ramorum* was inoculated alone, it rapidly colonized most of the green leaf area and persisted at this level throughout the 16 weeks of incubation (Figure 1). It did not effectively colonize brown, senesced leaves, though it could occasionally be recovered from a few pieces of some leaves. In contrast, *P. gonapodyides* colonized most of the area of both green and brown leaves in microcosms where it was inoculated (Figure 1). However, *P. gonapodyides* colonized brown leaves to a significantly lesser degree than green leaves when the leaves were exposed to inoculum in separate microcosms, while there was no difference between the colonization of green and brown leaves when they were maintained in the same microcosm (Figure 1). In combined inoculations of both *Phytophthora* species, *P. ramorum* was unexpectedly suppressed on both leaf types and the recovery of *P. gonapodyides* from

this treatment was identical to that of *P. gonapodyides*-only treatments (Figure 1). Reflecting these results, in both experiments with green and brown leaves, the interaction of inoculation and leaf type was highly significant ($p < 0.0001$, Tables S1 and S2).

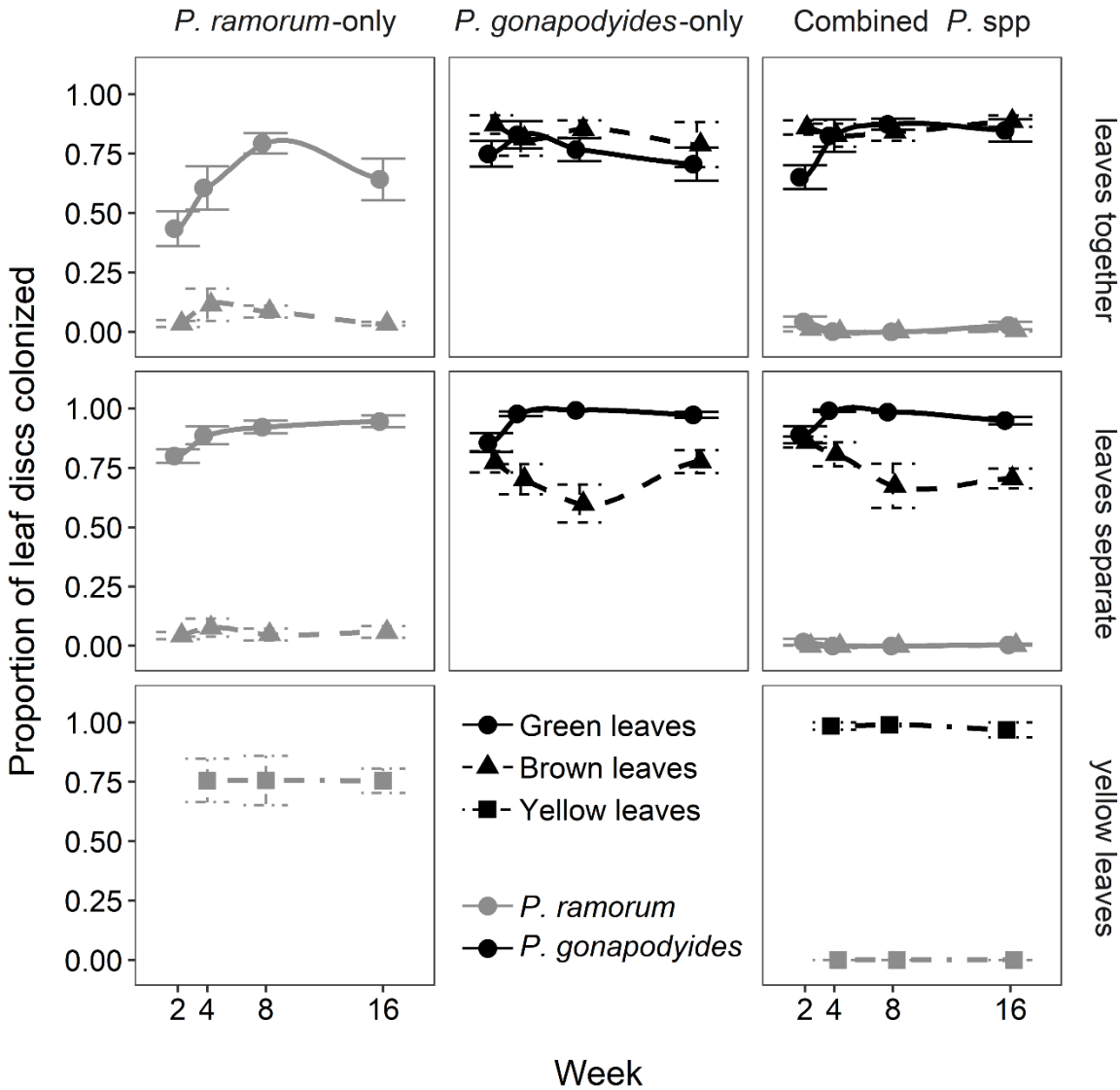

Figure 1. Proportion of green, brown and yellow leaves (designated by element shape and line type) colonized by *P. ramorum* or *P. gonapodyides* (designated by element and line shade)—determined as the proportion of leaf pieces colonized out of the total number sampled in "mosaic" isolations—for three different inoculum treatments (horizontal panels) at sampling intervals over 16 weeks of incubation in three different experiments (vertical panels). Two experiments included green and brown leaves, the first with both leaf types in the same microcosm and the second with each leaf type in different microcosms. One experiment included yellow leaves only with only *P. ramorum* and combined *Phytophthora* species inoculation treatments. Non-inoculated treatments are not shown, and results are averaged over stream water treatments which did not have a significant effect, except for the experiment with yellow leaves which used only sterile nutrient solution. Bars represent ± standard error, $n = 10$.

Though the difference was not significant in the overall model, in *P. ramorum*-only inoculated treatments where brown leaves were maintained separately from green leaves, *P. ramorum* colonized brown leaves at consistently higher levels in autoclaved water treatments compared with treatments with non-sterilized stream water added (0.094 and 0.030 mean fraction of leaf discs colonized, respectively). This difference was less apparent with green leaves (Supplemental Figure S2).

Nevertheless, there were no statistically significant differences between treatments based on stream water additions (Tables S1 and S2), and therefore, results are presented averaged over this factor.

In the experiment with yellow leaves, *P. ramorum*, when inoculated alone, colonized most of the leaf area and persisted at this level throughout the experiment, similar to the result with green leaves in other experiments (Figure 1). In combined *P. ramorum* and *P. gonapodyides* inoculations, *P. ramorum* was once again completely suppressed and *P. gonapodyides* colonized yellow leaves almost completely, at levels similar to its colonization of green leaves in both other experiments (Figure 1). The colonization of yellow leaves by *P. gonapodyides* in combined *Phytophthora* inoculum treatments was significantly higher than that by *P. ramorum* in *P. ramorum*-only inoculated treatments in this experiment, though both species colonized more than 70% of the leaf area. Thus, in the experiment with yellow leaves, only the effect of *Phytophthora* inoculation was significant ($p = 0.0159$, Table S3). In all experiments, both *Phytophthora* species colonized leaves rapidly, in most cases reaching maximum levels by four weeks, and persisted at these levels throughout the 16 weeks experimental duration. A slight increase in the level of colonization by both *Phytophthora* species was apparent in many cases from two to four weeks, though for brown leaves maintained in separate microcosms in the second experiment, levels appeared to actually decline after the second week. This contrast is reflected in the significant interaction of leaf type and collection week for this experiment ($p = 0.0135$, Table S2).

3.2. Sporulation

The isolation of *P. ramorum* or *P. gonapodyides* from California bay leaf disc baits deployed on the water surface in microcosms indicated the presence of zoospores. The sum of successful bait isolations for each treatment across the five replication blocks in the green and brown leaf experiments, and across four replications in the yellow leaf experiment, are presented in Tables 1–3. The recovery of *P. gonapodyides* from *P. gonapodyides*-only and combined *Phytophthora* inoculation treatments was from nearly 100% of baits throughout the duration of all experiments. The recovery of *P. ramorum* was more erratic, ranging from 40% to 90% of baits during the experiments with green and brown leaves. However, *P. ramorum* recovery from baits in microcosms that included green leaves and sterile rather than non-sterilized water was closer to 100%, excepting the second baiting of the second experiment, when *P. ramorum* was not recovered from most microcosms. *Phytophthora ramorum* was also rarely recovered by baiting from microcosms in the second experiment with only brown leaves, especially when excluding the first baiting, which was done a few days after inoculation. Consistent with this, brown leaves were colonized at very low levels by *P. ramorum*. Nevertheless, at 14 weeks, *P. ramorum* could still be recovered from several of these microcosms (Table 2). *Phytophthora ramorum* was recovered somewhat more frequently from sterile than non-sterile stream water treatment. Such an effect was not apparent for *P. gonapodyides*. Both *Phytophthora* species were recovered at nearly 100% from baits throughout the yellow leaf experiment which used sterile dilute nutrient solution only (Table 3).

Additionally, we baited microcosms for weeks after all leaf packets had been collected to see how long spores may persist in the absence of leaves. *Phytophthora ramorum* could be recovered from a few microcosms up to six weeks after all leaves were removed, but its frequency generally diminished rapidly. In contrast, *P. gonapodyides* could be recovered for up to 12 weeks after all leaves had been removed from microcosms, and was relatively frequent even six weeks after leaves were removed in the second experiment.

Table 1. Count of *P. ramorum* (*Pr*) and *P. gonapodyides* (*Pg*) recovery from single leaf disc baits deployed for three to seven days in a total of five microcosms per treatment (i.e., out of five possible colonization events per sampling, 35 total. Dash indicates not inoculated and not recovered) in the first experiment where green and brown leaves were maintained together in microcosms with either sterile (st) or non-sterile (nst) stream water added. Grey shading indicates results from after all leaves had been removed from the microcosms (126 days).

		wk	day	wk	day	wk	day	wk	day	wk	day	wk	day	wk	day	Total	
		7	52	10	71	16	111	18	123	19	133	20	141	22	151		
Water	Inoculum	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*
nst	*Pr*	3	-	3	-	4	-	3	-	0	-	1	-	1	-	15	-
st		5	-	5	-	5	-	3	-	3	-	2	-	0	-	23	-
nst	*Pg*	-	5	-	5	-	5	-	4	-	0	-	0	-	1	-	20
st		-	3	-	5	-	5	-	4	-	3	-	2	-	2	-	24
nst	*Pr* + *Pg*	0	5	0	5	0	5	0	5	1	3	0	0	0	0	1	23
st		0	5	0	5	0	5	1	3	0	2	0	0	0	1	1	21

Table 2. Count of *P. ramorum* (*Pr*) and *P. gonapodyides* (*Pg*) recovery from single leaf disc baits deployed for three to seven days in a total of five microcosms per treatment (i.e., out of five possible colonization events per sampling, 35 total. Dash indicates not inoculated and not recovered) in the second experiment where green and brown leaves were maintained in separate microcosms with either sterile (st) or non-sterile (nst) stream water added.. Grey shading indicates results from after all leaves had been removed from the microcosms (112 days).

			wk	day	wk	Day	wk	day	wk	day	wk	day	wk	day	wk	day	Total	
			0	2	10	69	14	100	17	117	19	134	22	153	28	193		
Water	Leaf	Inoculum	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*
nst	Green	*Pr*	2	-	2	-	5	-	1	-	0	-	0	-	0	-	10[a]	-
st			5	-	0	-	5	-	3	-	5	-	2	-	0	-	20	-
nst	Brown		5	-	0	1	2	-	0	-	1	-	0	-	0	-	8	1
st			5	-	0	-	1	-	0	-	0	-	0	-	0	-	6	-
nst	Green	*Pg*	-	5	-	5	-	5	-	4	-	4	-	5	-	2	-	30 [a]
st			-	5	-	5	-	5	-	5	-	4	-	5	-	1	-	30
nst	Brown		-	5	-	5	-	5	-	3	-	2	-	4	-	2	-	26
st			-	5	-	5	-	5	-	4	-	3	-	4	-	1	-	27
nst	Green	*Pr* + *Pg*	0	4	0	3	0	5	0	3	0	4	0	5	0	2	0	26 [a]
st			0	5	0	4	0	5	0	1	0	3	0	2	0	3	0	23

Table 2. *Cont.*

			wk	day	wk	Day	wk	day	wk	day	wk	day	wk	day	wk	day	Total	
			0	2	10	69	14	100	17	117	19	134	22	153	28	193		
Water	Leaf	Inoculum	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*
nst	Brown		0	5	0	5	0	5	0	4	0	1	0	4	0	1	0	25
st			1	5	0	5	0	5	0	4	0	1	0	4	0	1	1	25

a: Out of 34 total attempts in each row per *Phytophthora* species.

Table 3. Count of *P. ramorum* (*Pr*) and *P. gonapodyides* (*Pg*) recovery from single leaf disc baits deployed for three to seven days in a total of four microcosms per treatment (i.e., out of four possible colonization events per sampling, 28 in total. Dash indicates not inoculated and not recovered) with sterile water only in the yellow leaf experiment. Grey shading indicates results when all leaves had been removed from the microcosms (116 days).

	wk	Day	wk	day	wk	day	wk	day	wk	day	wk	day	wk	day	Total	
	8	54	12	85	14	97	15	102	17	119	20	138	25	178		
Inoculum	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*	*Pr*	*Pg*
Pr	3	-	4	-	4	-	4	-	4	-	1	-	0	-	20	-
Pr + *Pg*	0	4	0	4	0	4	0	4	0	4	0	4	0	4	0	28

3.3. Leaf Decomposition

In the experiment with green and brown leaves maintained in the same microcosm, only green leaves in microcosms with no *Phytophthora* inoculum decomposed at a slower rate than all other treatments (Figure 2). In fact, on average, they did not lose significant biomass throughout the 16 weeks. Leaves in all other treatments, including brown leaves in non-inoculated microcosms, decomposed at similar rates (Figure 2). The interaction of leaf type and *Phytophthora* inoculation was, therefore, a highly significant predictor in the model ($p < 0.0001$, Table S4). Estimated decay constants are listed in Table S5.

In the experiment where green and brown leaves were maintained in different microcosms, green leaves in microcosms with *Phytophthora* inoculum decomposed faster than all other treatments (Figure 2). In this experiment, all treatments with brown leaves and green leaf treatments with no *Phytophthora* decomposed at similar rates. Notably, in contrast to the other experiment, green leaves in non-inoculated treatments in this experiment did decompose over the 16 weeks, ultimately achieving a similar level of biomass loss as green leaf treatments with *Phytophthora* inoculum. Nevertheless, reflecting the difference in decomposition rate for green leaves in inoculated and non-inoculated treatments, the effect of the leaf type by *Phytophthora* inoculum interaction was significant in the model ($p = 0.0094$, Table S6). Estimated decay constants are listed in Table S7.

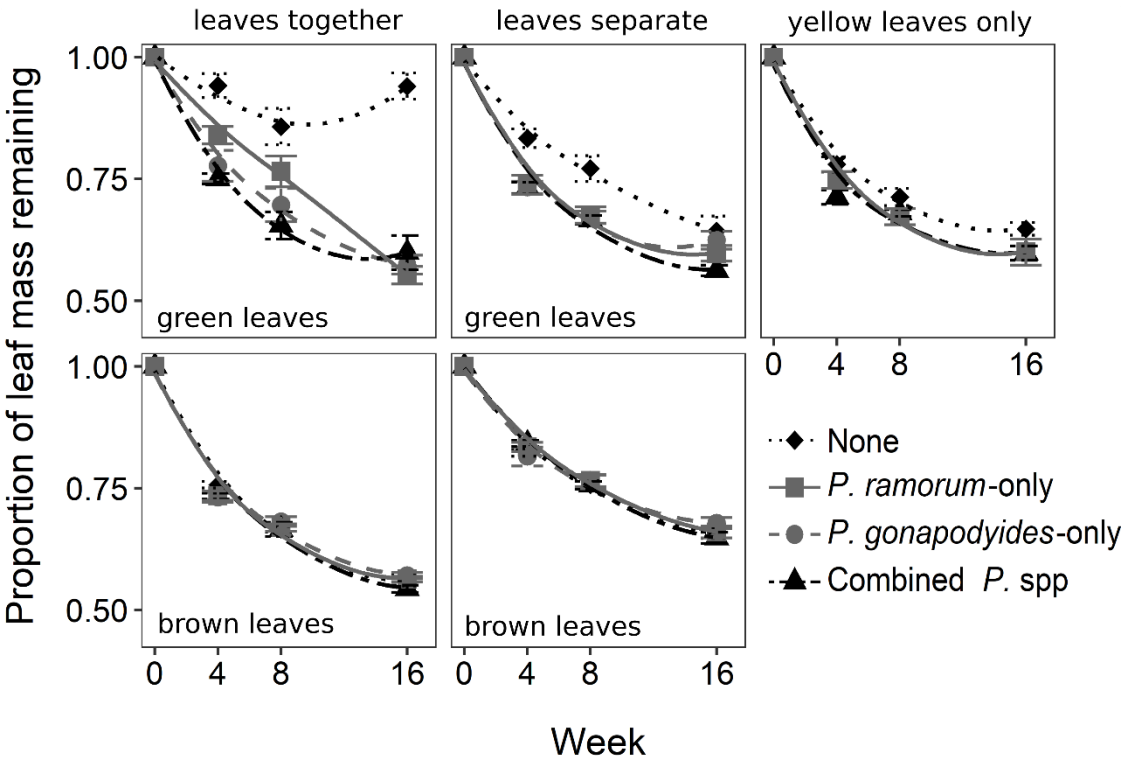

Figure 2. Leaf decomposition represented as a proportion of leaf mass remaining at sampling intervals over 16 weeks for green (top left two panels), brown (bottom panels) and yellow leaves exposed to three *Phytophthora* inoculum treatments and one non-inoculated control in three different experiments (vertical panels). Two experiments included green and brown leaves, the first with both leaf types together in the same microcosm and the second with each leaf type separate in different microcosms. One experiment included yellow leaves only with only *P. ramorum* and combined *Phytophthora* species inoculum treatments. Results are averaged over stream water treatments which did not have a significant effect, except for the experiment with yellow leaves which used only sterile nutrient solution. Bars represent ± standard error, $n = 10$.

In the experiment with yellow leaves only, leaves in the non-inoculated treatment decomposed at a slightly but significantly lower rate than *Phytophthora*-inoculated treatments ($p = 0.0292$, Figure 2, Table S8), of which the decomposition rates were not statistically different from one another (Table S9). The decomposition rate of yellow leaves in *Phytophthora*-inoculated treatments was similar to that of green leaves in *Phytophthora*-inoculated treatments of the second experiment with which it was essentially concurrent, though the results of the different experiments were not statistically compared.

4. Discussion

The goal of these experiments, broadly, was to better understand how the previously observed differences in trophic specialization between *P. ramorum* and *P. gonapodyides* [45], the latter as a representative of stream-resident clade 6 *Phytophthora* species, affected their ability to utilize different kinds of leaf litter available in streams. More specifically, we sought to determine if the previously observed decline of *P. ramorum* in green leaves decomposing in streams [45] was due to competitive displacement by saprotrophic organisms or due to an intrinsic inability of this pathogenic species to persist on colonized but decomposing leaf tissue, and to discover if the observed specialization of each species as pathogen or saprotroph would be consequential for the colonization of senescent or fully senesced leaves, a factor that has important implications regarding the prevalence of suitable leaf litter substrate for these organisms. Additionally, we wanted to test the contribution by each *Phytophthora* species to leaf decomposition and to determine if there was any difference depending on leaf senescence based on their differing trophic adaptations. While the inclusion of natural stream water in these experiments is an imperfect approximation of natural conditions, namely in excluding both shredder organisms and other microorganisms eliminated in the holding period, it had the potential to reflect the interaction of the inoculated *Phytophthora* species with bacteria, protozoa, fungal communities, and possibly micro-invertebrates that persisted in stream water. Though the effect of stream water treatment was not statistically significant in the models, a noticeably higher occurrence of *P. ramorum* on brown leaves in sterile stream water treatments compared with non-sterilized stream water additions (Supplemental Figure S2) and also higher detection of *P. ramorum* spores by baiting in sterile compared to non-sterile stream water treatments (Tables 1–3) both indicated that there was some difference between the two treatments. Though a much greater diversity of organisms likely influences this system under natural conditions, our previous research exposing leaves in natural streams demonstrated both *P. ramorum* and clade 6 *Phytophthora* species effectively colonize California bay leaves under natural conditions [45].

As expected, based on previous work [45], both *Phytophthora* species rapidly colonized more than 60% of the leaf area of green leaves in both experiments. That *P. ramorum* also persisted on green leaves at high levels for the entire 16 weeks despite the loss of approximately 40% of leaf biomass stands in contrast to our previous findings where its colonization of leaves peaked within a few weeks after exposure in natural streams, but then rapidly dropped to very low levels as colonization by clade 6 *Phytophthora* species rose and persisted at high levels [45]. This is evidence that the reduced recovery of *P. ramorum* from green leaves in natural streams as decomposition progressed was due to displacement from saprotrophic organisms like clade 6 *Phytophthora* species. Unfortunately, *P. ramorum* was completely suppressed from colonizing leaves in combined inoculations with *P. gonapodyides* and it could not be determined if the pattern observed in field experiments would occur under these simulations when both species were present. The suppression of *P. ramorum* colonization of green leaves in combined *Phytophthora* inoculations—consistent across all three experiments—was surprising because both species were effective at colonizing leaves when inoculated alone. One explanation could be that sporulation of *P. gonapodyides* from mycelial mats occurred earlier than that of *P. ramorum* and that the latter was therefore precluded from leaves because in all experiments, full colonization of green leaves by *P. gonapodyides* occurred very rapidly. Indeed, in the first experiment, colonization of *P. gonapodyides* occurred more rapidly on green leaves than that of *P. ramorum* (Figure 1). However, baiting two days after inoculation in the second experiment showed that *P. ramorum* spores were

active in the microcosms where it was inoculated alone, but almost absent in the combined inoculation microcosms. This suggests that the presence of *P. gonapodyides* itself may have suppressed sporulation by *P. ramorum*. The rapid leaf colonization by *P. gonapodyides* in these microcosms also contrasts with the slower colonization that was observed in natural streams [45] and may be an artifact of high inoculum loads and the relative abundance of substrate. The aim of these experiments was to characterize the capacity of each organism for growing and reproducing from each type of leaf rather than estimating typical colonization and decomposition in streams. Though logistically more difficult to prepare and standardize for an experiment of this magnitude, using sporangia or zoospore inoculum rather than mycelial mats may overcome the problem of uneven inoculum activation, the success of which we have experienced in smaller scale experiments [45]. Alternatively, the use of colonized plant tissue (e.g., leaf discs) instead of mycelial mats as a source of inoculum may also produce a different outcome from the suppression of *P. ramorum* that we found with this approach in mixed inoculations. Interestingly, the kind of succession observed in field experiments did occur in a few control microcosms into which both *Phytophthora* species were accidentally contaminated (data not shown). However, the limited occurrence and unknown relative quantity of original inoculum precluded more substantial evaluation. In any case, the suppression of *P. ramorum* sporulation in treatments where *P. gonapodyides* was present raises the question of what mechanism was responsible for the effect. It also furthers the impression that *P. gonapodyides* and other clade 6 *Phytophthora* species may have a moderating effect on the presence of *P. ramorum* in streams.

The green leaves that we used were of mature cuticle and collected in midwinter and late summer for the first and second experiments, respectively. While some seasonal variation in susceptibility to *P. ramorum* infection has been reported in California bay leaves [60,61], the physical and chemical properties of mature leaves have also been reported to be relatively consistent throughout the year [62]. Our results were similar for both experiments, and therefore, any variation in the leaves was overcome by experiment factors.

The extensive colonization of brown leaves by *P. gonapodyides* and their limited colonization by *P. ramorum* is consistent with previous work where we showed that the former is a competent saprotroph while the latter is relatively ineffective at colonizing dead tissue [45]. A significant discovery in this work was that *P. ramorum* colonized yellow, senescent leaves that were still fresh and had an intact cuticle to nearly the same degree as it did green leaves. At this stage, though chloroplasts and most of the protein content are gone from leaves, the cells are expected to be still alive, while in brown leaves that have dried the cells are no longer biologically active [30,43,44]. In fact, colonization of the yellow leaves by *P. ramorum* was not quite as extensive as its colonization of green leaves in the second experiment, which ran more or less concurrently and in which green and brown leaves were maintained in separate microcosms (Figure 1), though the difference between the separate experiments was not analyzed statistically. Though green leaves are shed into streams as a relatively low proportion of total litter, yellow leaves, often shed directly into streams from trees, constitute a much greater proportion of leaf litter in streams (Aram, personal observation, see also [29]). This indicates that a great proportion of leaf litter in the streams is suitable for colonization by *P. ramorum*, and conforms to the regular recovery of this pathogen from natural leaf litter [45]. Furthermore, the degree of colonization of yellow leaves by both *Phytophthora* species remained persistent throughout the 16 weeks, as with green leaves in the other experiments, suggesting that the same kind of succession may be expected in these leaves as seen with green leaves in natural streams [45].

Also consistent with previous findings with leaves colonized in naturally infested streams [45], leaves colonized by both *Phytophthora* species were generally conducive to sporulation as detected by baiting from the microcosms. *Phytophthora gonapodyides* was consistently recovered from *P. gonapodyides*-only and combined *Phytophthora* species inoculation treatments where it had colonized all green and brown leaves at all sampling points. The results from baiting of *P. ramorum* spores from microcosms were less regular, but nonetheless, mostly successful from microcosms containing colonized green or yellow leaves and occurred minimally from microcosms containing brown leaves

which were colonized at only very low levels. The relatively less frequent recovery of *P. ramorum* by baiting from microcosms with non-sterilized stream water, not observed for *P. gonapodyides*, may be the consequence of *P. ramorum* not being well adapted to sporulation in biologically active aquatic environments or relying on different environmental signals. Nevertheless, these results confirm that both of these *Phytophthora* species can sporulate from colonized, decomposing leaves, whether green, yellow or brown leaves. Furthermore, at least under these conditions, their spores persisted for weeks and even months after any visible substrate was available, though the effect occurred more definitively and for longer with *P. gonapodyides*. As *P. gonapodyides* is not known to produce long-term survival structures, the question arises of how *P. gonapodyides* persisted so long in the microcosms in the absence of leaves. This observation also stands in contrast to our successful elimination of *Phytophthora* spores from original stream water collections simply by holding the water at cool temperatures for approximately three weeks. The observed persistence of spores of both *Phytophthora* species may be the result of an abundance of zoospore cysts due to the compact nature of the microcosms, or perhaps because the spores originated from propagules that would not have been suspended in the water column of the flowing streams.

While oomycetes have been acknowledged as decomposers in aquatic environments until recently they have primarily been regarded as acting on non-cellulosic detritus such as insect and animal tissue [63]. As most *Phytophthora* species are known as plant pathogens, the recent evidence that they may also degrade plant tissue in detritus is not surprising [64–66]. Parasitism is considered an early characteristic in the evolution of oomycetes, [67], but the possible evolution of a saprotrophic lifestyle from parasitic precursors has been considered for fungi and oomycetes [67,68]. Clade 6 *Phytophthora* are known to be opportunistic pathogens [2,69–71]. Stradling saprotrophic and parasitic lifestyles, stream-resident *Phytophthora* may play an important role in the early breakdown of leaves and vegetative matter that still contain living cells. As facultative pathogens, [2,69–71] clade 6 *Phytophthora* species can enter living cells and open intact tissues to further colonization by other saprotrophic organisms with less ability to penetrate living tissue. This is analogous to the paradigm of 'conditioning' of vegetative litter by pioneer microbial species [29,30], though in this case with respect to secondary saprotrophic microorganisms that could not on their own overcome physical and chemical protections still present in senescent but still alive leaf tissue. Our results were consistent with this hypothesis, as green leaves decayed more slowly in the absence of *Phytophthora*. It is uncertain why in the first experiment green leaves in the treatments with no *Phytophthora* inoculation decomposed very little over the entire 16 weeks of the experiment. In this experiment, both green and brown leaves were maintained together in microcosms, and it is possible that leachate from the leaves, particularly the brown leaves, may have had an inhibitory effect on some microorganisms. In the second experiment, leaves were leached prior to being deployed in the experiment, and also green and brown leaves were kept in separate microcosms. Green leaves in non-inoculated controls in the second experiment lost biomass to a degree ultimately similar to that of inoculated treatments, albeit at a slower rate. This indicates that other organisms were present that could initiate the decomposition of green leaves through the presence of *Phytophthora* accelerated it. We attempted additional isolations from some samples of leaves on acidified potato dextrose agar medium and found that the leaves in both controls and inoculated treatments were generally well colonized by a multitude of fungi (data not shown). The fact that similar fungi occurred on leaves from microcosms prepared with both sterile and non-sterilized stream water suggests that many of these fungi were present on the leaves before entering streams as leaf litter (e.g., [72]). A diversity of fungi have been reported from bay leaves in coastal California forests [73]. Additionally, overall there were no differences in decomposition rates between treatments with sterile or non-sterilized stream water added. Decomposition was also similar for leaves colonized by either *Phytophthora* species, indicating that, though *P. gonapodyides* is a better adapted saprotroph, both species had a similar effect on the decomposition of live, green and yellow leaves. This would be consistent with *Phytophthora* having the effect of opening integral tissue to colonization by other saprotrophs that then push decomposition forward. Finally, it is interesting that the presence of fungi in these

leaves did not affect the persistence of *P. ramorum* throughout the experiments, suggesting that they are using different resources and that the successive displacement of *P. ramorum* in previous work may be specific to competition with other *Phytophthora* species or similar organisms. Under natural conditions, leaves would be exposed to a greater diversity of organisms, including other oomycetes such as *Phytopythium* species [74].

As *P. gonapodyides* can colonize dead leaf tissue, it could be expected that it would contribute to leaf decay in brown leaves as well. This was not observed, as loss of biomass in brown leaves was the same in all treatments unaffected by *Phytophthora* colonization. The fact that *P. gonapodyides* substantially colonized brown, senesced leaves, but did not increase the rate of biomass loss raises the question as to what resources the organism uses in this substrate. Though biomass loss is a useful measure of decomposition [29,53], it does not offer a complete picture and other measures, such as changes in leaf toughness or chemical properties may offer a fuller picture of decomposition [29,75] that could account for the effects of *Phytophthora* colonization. Moreover, decomposition of brown leaves proceeded more slowly in the second experiment than the first. This may be due to lower nitrogen and other nutrient availability both because in the first experiment green and brown leaves were maintained together in microcosms and also that in the second experiment, the leaves were leached prior to being introduced into microcosms at the start of the experiment [28]. This may also be the reason that colonization of brown leaves by *P. gonapodyides* was significantly less than that of green leaves when leaves were kept in separate microcosms, while the levels were similar when leaves were maintained in the same microcosms. Another possibility is that sporulation from green leaves allowed greater colonization of brown leaves where the leaves were kept in the same microcosm.

Our results demonstrate that green and yellow California bay leaves are suitable substrates for the growth, colonization, and sporulation of *P. ramorum* in streams where they constitute a significant proportion of vegetative litter, they likely play an important part of supporting the inoculum load in streams. Yellow leaves resemble green ones in that, in contrast with brown leaves, they have an intact cuticle, and their cells are essentially still alive. California bay leaves infected by *P. ramorum* have been shown to senesce and abscise from trees more frequently than uninfected leaves [37]. In infested forests, a great portion of senescent leaves probably enters the stream already colonized by *P. ramorum* [37,45]. As leaves that fall into the water do not dry out, their cells likely remain alive for an extended period, allowing further colonization by *P. ramorum*. However, stream resident clade 6 *Phytophthora* species also compete for this substrate and may limit the extent to which *P. ramorum* can grow on, persist, and reproduce from them [45]. As dry, brown, senesced California bay leaves begin to make up a greater proportion of leaf litter in late summer and fall, the ability of clade 6 *Phytophthora* species to exploit these, while *P. ramorum* cannot, may be one explanation for why the latter is recovered less regularly and with lower frequency from these and other California streams in the fall and early winter [46]. Moreover, as the summer progresses, green and yellow leaves will be more decomposed and less suitable for *P. ramorum*. The warming of streams late in the summer may additionally favor clade 6 *Phytophthora* species that are known to have generally higher optimal growth temperatures than most other species [5]. We maintained temperatures constant for experimental purposes, but the persistence and sporulation of these *Phytophthora* species, and *P. ramorum* in particular, may be significantly affected by temperature fluctuations and extremes.

We have isolated *P. ramorum* and clade 6 *Phytophthora* from leaf litter of other tree species in naturally infested streams, including leaves of coast redwood, madrone, white alder, big leaf maple, and coast live oak (authors' unpublished data). Occasionally, we have found portions of other submerged riparian plants, such as chain fern (*Woodwardia fimbriata* Sm.) or elk clover (*Aralia californica* S. Watson), to be colonized (authors' unpublished data). It is well-established that California bay leaves are an optimal substrate for *P. ramorum*, and though clade 6 *Phytophthora* species are known from a great variety of vegetative litter, it is uncertain how conducive other vegetative litter would be to survival and sporulation of either species. Stamler et al. [20] recovered primarily clade 6 and 9 *Phytophthora* species from rivers in the southwestern USA using leaves of *Salix* and *Populus* species, common as

riparian trees, as bait. It would be expected that natural leaf litter in such ecosystems would also harbor these organisms. Themann et al. [76] recovered primarily *P. gonapodyides* but also *P. cinnamomi* from vegetative litter in sediments in an irrigation reservoir. Therefore, leaf and other vegetative litter should be considered as potential sources of *Phytophthora*, including pathogenic species, whether they are found in natural streams or other surface waters. Alternatively, the suitability of local vegetation may be a determinant of what *Phytophthora* species become established or prominent in streams.

5. Conclusions

With these studies, we have demonstrated that the trophic specializations of *Phytophthora* species in coastal California streams determine what leaf litter is available to them, but that nevertheless, suitable leaf litter is available throughout much of the year for both *P. ramorum* and clade 6 *Phytophthora* species. The role of stream resident *Phytophthora* species in leaf decay is probably one analogous to "conditioning" of fresh leaf litter (i.e., opening biologically integral tissues), essentially accelerating the earliest stages of decomposition. Nevertheless, they continue to persist and sporulate even as leaves become substantially decomposed. Green and yellow California bay leaves were similarly conducive to colonization and sporulation by both *P. ramorum* and *P. gonapodyides*, and the effects of both *Phytophthora* species on the decomposition of these leaves were similar. *Phytophthora ramorum* could not, however, colonize brown, biologically dead leaves, and though *P. gonapodyides* colonized brown leaves, it did not contribute to leaf decomposition as measured by loss of biomass. These studies expand the current knowledge about the ecological role of *Phytophthora* in streams.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/10/5/434/s1, Figures S1 and S2, Tables S1–S9.

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