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Insect-Associated Bacterial Communities in an Alpine Stream

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Abstract

The roles of macroinvertebrate and microbial communities in stream ecosystems are recognized to be important to energy flow and nutrient cycling. While the linkages of these major groups of aquatic organisms has not been thoroughly investigated, determining how they interact is particularly important for understanding the mechanisms and potential evolutionary relationships that contribute to ecosystem processes, such as organic matter decomposition. We evaluated the microbiomes of several aquatic macroinvertebrate species differing in trophic ecology and belonging to different functional feeding groups at two sites along an Italian Alpine river with different elevation and environmental characteristics, one located above the tree-line and the other in a forested environment. We found that the internal microbial communities of the different insect species significantly varied in taxonomic and functional composition and could be used to classify samples to both species and environment. We demonstrated that functional differences existed between the microbiota of different macroinvertebrate species with variable feeding behaviors, and that species differences were more important, in this context, than environmental or habitat conditions. These results provide new information on how the microbiomes of macroinvertebrates may potentially be influenced by their hosts and habitat conditions in Alpine streams.

Introduction

There has been limited study of entire microbial communities associated with aquatic macroinvertebrates, and this is especially true in high altitude and high gradient mountain streams. Evidence from other systems suggests that the internal microbial communities, or microbiota, of insects and other invertebrates have important functional effects on both their biology and ecology (Douglas 2015; Henry et al., 2015; Moran & Telang 1998). These studies also show that the internal microbiomes may have co-evolved with certain species (e.g., ants) (Hooper et al., 2012; Moran & Telang 1998; Russell et al., 2009), providing important functional roles to the fitness and dispersal of many species. In addition, research has shown that the microbes of decaying organic matter (Benbow et al., 2019), in the form of plant (Cummins et al., 1973; Eggert & Wallace 2007; Moore et al., 2004) and animal (Pechal & Benbow 2016; Pechal et al., 2013) detritus, are acquired through feeding activities and may be transferred through insect developmental stages (e.g., larvae to pupae to adults) (Hocking & Reimchen 2006; Pechal et al., 2019; Weatherbee et al., 2017). In freshwater ecosystems microbial communities contribute to the decomposition of autochthonous and allochthonous organic matter (Baldy et al., 1995; Webster & Benfield 1986), and are known to vary along the watershed continuum (Savio et al., 2015), likely responding to riparian forest conditions, hydrological regimes and biotic interactions (Besemer et al., 2013; Widder et al., 2014) in ways that mediate the quality and quantity of organic matter that is transported downstream. However, how the internal microbial communities of aquatic macroinvertebrates contribute to these processes remains largely unknown.

Organic matter subsidies vary in quantity and typology along the length of watersheds (Cummins 1974; Vannote et al., 1980), and are intimately linked to the structure and diversity of

72 riparian and basin vegetation. Small order streams draining forested watersheds have significantly
73 higher allochthonous organic matter inputs (i.e., litterfall) than streams draining unforested areas
74 (Golladay 1997; Tank et al., 2010). Since the formulation of the River Continuum Concept
75 (Vannote et al., 1980), stream ecology has adopted, modified and refined (Sedell et al., 1989;
76 Statzner & Higler 1985; Winterbourn et al., 1981) a theoretic framework in which terrestrial and
77 aquatic ecosystems are intrinsically linked so that biological, physical, and chemical changes can
78 be predicted along a longitudinal gradient. Under this framework, mountainous lotic systems
79 assume great interest in understanding how organic matter is processed at the upper elevations of
80 high gradient watersheds which harbor unique sets of ecological processes and specialized taxa
81 (Ward & Saltz 1994).
82
83 Mountainous, low-order streams are distinctive systems characterized by cold, highly
84 oxygenated and turbulent water, steep gradients, coarse substrata, low channel stability and limited
85 nutrient availability (Hieber et al., 2005). Among these systems, the tree line defines separate areas
86 of the riparian zone with different limiting factors for plant growth and thus species composition
87 (Figure 1A). This abrupt change in riparian conditions is important (but largely uninvestigated in
88 Alpine regions) in influencing solar radiation and quantity and quality of terrestrial organic matter
89 subsidies. For example, below the tree line most energy inputs are derived from allochthonous
90 non-living coarse particulate organic matter (CPOM), mainly terrestrial leaves (Tank et al., 2010),
91 while above catchments have scarce terrestrial vegetation, and consequently reduced input of
92 allochthonous organic matter. Aquatic macroinvertebrate communities are known to respond both
93 taxonomically and functionally to changes in allochthonous CPOM from the riparian forests
94 (Cummins & Klug 1979; Doretto et al., 2016; Merritt & Cummins 2006; Vannote et al., 1980).

One approach to investigate the linkages between allochthonous subsidies and associated biotic processing has been to evaluate functional groups of aquatic macroinvertebrates that process and consume organic matter differently (Cummins 2016; Cummins & Klug 1979; Merritt & Cummins 2006; Straka et al., 2012). Aquatic macroinvertebrates use a range of feeding strategies to obtain nutrients, and as such, display morphological and behavioral traits which can be used to classify their feeding behaviors into broad groups. These groups range from shredders, which feed directly on allochthonous inputs such as leaf materials (and microbes associated with these resources), to scrapers, which feed predominantly on microbial biofilms present on substrates, and predators. It has been shown that the gut bacterial communities of aquatic macroinvertebrates change in response to different food sources, with most studies using culture-based survey approaches (Kaufman et al., 2000; Lawson et al., 1984); however, more comprehensive descriptions of how the entire gut microbial community, using recent genomic sequencing technologies, responds to changes in allochthonous CPOM inputs has been less studied (Pechal & Benbow 2016; Yun et al., 2014).

The overall goal of this study was to describe the internal microbiota of aquatic macroinvertebrate species at two elevations associated with distinct environmental characteristics (mainly related to elevation and the presence or absence of riparian forest cover) along an Alpine stream in Italy. We predicted that the internal microbiota of aquatic insects would differ, in part based on their elevational locations, with lower microbial diversity at the higher elevation site receiving less diverse CPOM, but that this difference would be mediated by individual species differences.

Materials and Methods

Study Location

Aquatic insects were collected at two sampling locations along the Po River, the longest Italian lotic system, which originates from a spring below the northwest side of the Monviso mountain, in the Cottian Alps of north-western Italy (Figure 1B). Pian della Regina (Alpine prairie) was the high elevation location at 1750 m above sea level (m.a.s.l.) and above the tree line of the drainage basin. Here the stream was an open system flowing across a plain of glacial origin, characterized by extensive Alpine meadows pointed by large erratic boulders and very few, scattered *Larix decidua* Mill., 1768. Riparian vegetation was composed almost exclusively of herbaceous species, Poaceae and Ericaceae. Within stream substrata were homogeneous and composed mainly by coarse elements (approximately 50% boulders, 40 % cobbles, 10% gravel/sand) (Figure 2A). Ostana (Forest) was the downstream, lower elevation location (971 m.a.s.l.) with forested riparian zones and slopes dominated by a mixed broadleaf forest containing *Fagus sylvatica*, *Acer* sp., *Fraxinus excelsior*, and *Alnus glutinosa*. Within stream substrata was similar to the Alpine prairie location and composed mainly of coarse elements (approximately 30% boulders, 50% cobble, 20% gravel/sand) (Figure 2B). These locations were selected by *a priori* knowledge of the aquatic macroinvertebrate taxa previously reported (Doretto et al., 2017; Fenoglio et al., 2015) where we could sample species belonging to different functional groups.

Sample Collections

At each location, selected aquatic insect taxa were collected on a single occasion, to minimize temporal variation in gut contents and environmental variables. On 23 November 2017, a season in which biodiversity and invertebrate abundance are generally highest in these ecosystems (Doretto et al., 2017; Fenoglio et al., 2015), benthic insects were hand collected by visually searching and turning over stream substrata. Five species that belong to the four most important functional feeding groups in mountain systems were collected: ~~for scrapers~~ the mayfly *Epeorus alpicola* (Eaton, 1871) (Ephemeroptera: Heptageniidae) belongs to the scraper FFG, and ~~for shredders~~ the crane fly *Tipula* (*Tipulidae*) *maxima* Poda, 1761 (Diptera; Tipulidae) belongs to the ~~.~~ shredder FFG. However, we could not find the same species of predator at both locations, so two species of Systellognathan Plecoptera were used for microbiome characterizations: *Dictyogenus alpinus* (Pictet, 1841) (Perlodidae) for the forested location and *Perla grandis* Rambur, 1842 (Perlidae) for the Alpine prairie location. No filterer species were collected at the alpine prairie location, while *Hydropsyche* sp. (Trichoptera: Hydropsychidae) were collected at the forested location.

All specimens were immediately preserved in 95% molecular grade ethanol within sealed glass vials for laboratory identification under a stereomicroscope using regional dichotomous keys (Belfiore 1983; Fochetti & Tiernod e Figueroa 2008; Moretti 1983; Rivosecchi 1984) and then weighed. This preservation approach was based on our previous success with describing the microbiomes of aquatic and terrestrial macroinvertebrates (Benbow et al., 2017; Pechal & Benbow 2016; Pechal et al., 2019; Receveur et al., 2018; Weatherbee et al., 2017). At the time of sampling, physical and chemical parameters were measured using multiparametric probes (physio-chemical properties [Quanta, Hydrolab] current [Mod RHCM, Idromar]) as well as organic matter and

nutrients according to Italian standard methods of the Agenzia per la Protezione dell'Ambiente e per i servizi Tecnici - Istituto di Ricerca sulle Acque Consiglio Nazionale delle Ricerche (APAT-IRSA 2003).

Nucleic Acid Isolation and Bioinformatic Data Processing

To limit the influence of microbes present on the external surfaces of the insects, after preservation and immediately prior to nucleic acid isolation, all samples were surface decontaminated using a 10% hypochlorite wash followed by a triple rinse in sterile water as previously described (Ridley et al., 2012), and as we have done in previous work with aquatic macroinvertebrates (Pechal & Benbow 2016; Receveur et al., 2018). Following surface decontamination, samples were homogenized using sterile pestles as previously described (Pechal & Benbow 2016; Receveur et al., 2018). Briefly, DNA extraction was performed using the Blood and Tissue DNA kit (Qiagen®) with the addition of lysozyme (15 mg ml^{-1}, Invitrogen) during the lysis step before being quantified fluorometrically using a Qubit 2.0 (Grand Island, NY, USA) and a dsDNA High Sensitivity Assay Kit (Invitrogen). All DNA preparations were stored at -20°C until library preparation. Library preparation and sequencing (2 x 250 bp paired-end reads) was performed by the Michigan State University Research Technology Support Facility on an Illumina MiSeq platform following previously described methods (Caporaso et al., 2011). Variable region 4 (V4) of the 16S rRNA gene was amplified using indexed primers 515f and 806r (5′-GTGCCAGCMGCCGCGGTAA -3′, 5′- GGACTACHVGGGTWTCTAAT -3′) as described previously (Caporaso et al., 2011; Claesson et al., 2010; Kozich et al., 2013). Demultiplexing and base calling were performed using Bcl2fastq (v 2.19.1, Illumina) and RTA (v 1.18.54, Illumina).

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The raw sequencing reads were quality filtered using QIIME 2 (v 2018.11) using default settings (Bolyen et al., 2018). DADA2 was used to filter samples and remove low quality reads as well as chimeric sequences and other artifacts commonly present in Illumina data (Callahan et al., 2016). After singletons and amplicon sequencing variants with an abundance lower than 0.0005% were removed, a Naïve Bayes classifier was trained using the region amplified by the primers (515f, 806r, 250 bp) and the Greengenes database (v 13.8) at a 99% confidence level before being used to assign taxonomy using default settings in QIIME 2 (Bokulich et al., 2018). Reads mapped to mitochondria or chloroplast were removed. A rooted phylogenetic tree, created using all remaining sequence variants and default settings in QIIME 2, FastTree (v 2)(Price et al., 2010), and MAFFT (v 7)(Katoh & Standley 2013) was used in calculating a phylogenetic diversity metric [Faith's phylogenetic diversity (Faith's PD)]. Both Faith's PD and Shannon diversity were calculated using defaults settings in QIIME 2 (Faith & Baker 2006). To evaluate the functional community differences, PICRUSt 2 (https://github.com/picrust/picrust2) (Langille et al., 2013) was used to assign filtered sequencing reads to functional orthologs [Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO)] using the mp hidden-state prediction method (Louca & Doebeli 2017). Sequences files for this study have been deposited in the NCBI database under the accession number PRJNA547724.

Statistical Analyses

Differences in macroinvertebrate mass between sampling location met the assumptions of normality and were tested with t-tests in R (v 3.5.2) (2013). Differences in the relative abundance of bacterial taxa between groups at the phylum and family level were tested using Kruskal-Wallis and Mann-Whitney tests in R with FDR corrections to account for multiple comparisons. To

identify taxa which were differentially abundant at the genus level and test how well a model was able to classify samples to group (species or location), a machine learning algorithm (Random Forest) was used. Rather than test for differential abundances in all genera present (> 100), only the top indicators for each comparison were evaluated to limit the potential for spurious conclusions. The importance of an indicator in a Random Forest model is determined by how much removing that taxon from a model decreases the overall accuracy. The ten most important genus level indicators (determined by mean decrease GINI score and mean decrease accuracy) used by the models to classify samples to group were tested for differences using Kruskal-Wallis and Mann-Whitney tests in R. The random forest model was implemented using default settings in the RandomForest package (1000 trees, v 4.6-14) (Liaw & Wiener 2002). Alpha diversity metrics (Faith's PD and Shannon diversity) were compared using Kruskal-Wallis tests with a FDR correction for multiple comparisons. Beta diversity and dispersion (taxonomic and functional) were compared between site and species using PERmutational Multivariate Analysis Of Variance (PERMANOVA, Jaccard distance, 999 iterations) tests implemented in the vegan package (v 2.5-4) (Oksanen et al., 2015). Differences in beta diversity were visualized using Principle Coordinate Analysis (PCoA) plots and shown with ellipses representing 95% CIs for the mean of each group. Data were visualized using a combination of ggplot2, ggpubr, and phyloseq packages (Kassambara 2017; McMurdie & Holmes 2013; Wickham 2016) with all code used in analysis available at https://github.com/BenbowLab/AlpineStreamMicrobiome.

Results

Stream Conditions and Macroinvertebrate Communities

231 The forest sampling location had a lower mean temperature (10.5 vs 12.8 °C), higher
232 conductivity (132 vs 98 μS cm^{-1}) and lower dissolved oxygen (9.38 vs 10.2 mg L^{-1}) than the Alpine
233 prairie location while other parameters measured were similar (Table S1). A total of 26 samples
234 were used for sequencing analysis (Table 1). No *Hydropsyche* sp. were collected at the Alpine
235 prairie. Only *P. grandis* was collected from the Alpine prairie location while *D. alpinus* was
236 collected from forested location (provided in the results but no group with less than three
237 individuals was included in statistical analyses). The average mass of *E. alpicola* was nearly
238 double (22.7 ± 3.86 [SE] mg vs 11.6 ± 4.0 mg) at the forest location ($t = 4.22$, $P < 0.001$), while
239 there was not a significant difference in *T. maxima* mass between locations ($t = 1.81$, $P = 0.21$;
240 Figure 3A).
241
242 *Internal Microbial communities*
243 From the 26 samples used for sequencing, a total of 809,647 reads were obtained after
244 filtering, representing 2,420 amplicon sequence variants. To limit bias due to differing read sizes,
245 samples were rarefied to 3,000 reads per sample (Figure S1). The three most abundant phyla across
246 all samples were Proteobacteria (51.9% ± [SE] 4.3), Bacteroidetes (17.5% ± 2.8), and Firmicutes
247 (13.3% ± 3.4), representing 83% of the total communities. As no statistically significant
248 differences in relative taxa abundance due to environment (i.e., location) were observed at the
249 phylum or family level between samples of the same species (Kruskal-Wallis, $P > 0.05$, Figure
250 3B), the two locations were combined to investigate what taxonomic differences between *E.*
251 *alpicola* and *T. maxima* were conserved at both sites. The relative abundances of Proteobacteria,
252 Tenericutes, and Planctomycetes were significantly higher in *E. alpicola* than *T. maxima* while
253 Bacteroidetes and Firmicutes were significantly more abundant in *T. maxima* (KW, $P > 0.05$,

Figure 3C, Table S2) At the family level, there were 13 bacterial families (greater than 1% of total abundance) that were differentially abundant between *E. alpicola and T. maxima* (KW, $P < 0.05$, Figure 3D, Figure S2, Table S3).

To identify important genera that were differentially abundant, rather than testing for differences in every genus (N = 170), a random forest modeling approach was used to determine the top ten indicators of each group, which would then be tested further. Modeling was able to correctly classify which location a sample came from, regardless of species, with an Out Of Bag (OOB) error rate of 3.85% at the genus level (one sample from the Alpine prairie site misclassified as forest). The ten most important genera for classification (determined by Mean Decrease Gini score and Mean Decrease Accuracy) were then tested using Kruskal-Wallis tests to determine if they were differentially abundant between locations. None of the top ten indicators using either ranking method were significantly different between locations (KW, $P > 0.05$, Table S4, Figure S3, Figure S4).

To determine if modeling could predict species, regardless of location, and identify differentially abundant genera, samples from the two locations were combined and tested similarly as above. The random forest model was able to predict species (*P. grandis* not included in model, N = 2) with an error rate of 8.33% (two *Hydropsyche* sp. misclassified). All of the top ten predictors using both ranking methods were significantly different among species (KW, $P < 0.001$, Figure S5, Table S5, Table S6) with multiple comparisons (Mann-Whitney, FDR correction, $P < 0.05$) shown in Figure 4.

Bacterial community diversity

Forest *E. alpicola* bacterial communities were more phylogenetically diverse (9.05 ± [SE] 0.7, Faith's PD: KW, $\chi^2 = 6$, P = 0.014) than *E. alpicola* from the Alpine location (4.73 ± 0.26), while Shannon diversity was not significantly different between locations (KW, $\chi^2 = 2.94$, P = 0.086). For *T. maxima*, diversity was not different between locations for either alpha diversity metric (KW, P > 0.05). Comparing species at the forest location, *T. maxima* and *Hydropsyche* sp. displayed similar levels of bacterial diversity while having significantly higher Shannon (Mann-Whitney, P < 0.05) and phylogenetic diversity (MW, P < 0.05) than *E. alpicola* (Figure 5A, Figure 5B). A similar pattern was observed at the prairie location with *T. maxima* and *D. alpinus* having significantly higher diversity than *E. alpicola* according to both metrics (MW, P < 0.05).

Differences in beta diversity was visualized using PCoA plots and compared with PERMANOVA tests using Jaccard distance. Species, location, as well as their interaction impacted beta diversity, with species having the strongest effect (PERMANOVA, P < 0.01, Table 2, Figure 5C). As the interaction between location and species significantly influenced beta diversity, the effects of species were investigated separately for each location. At both the forest (*E. Alpicola, T. maxima, and Hydropsyche* sp.) and prairie (*E. alpicola T. maxima, and D. alpinus*) locations, all species within a location had significantly different microbial communities from each other (PERMANOVA, P < 0.05).

Functional community composition

Due to the reliance on high-quality gene annotations for predicting individual functional pathways, which are lacking for many poorly characterized environmental sample types (Langille et al., 2013; Radivojac et al., 2013), predicted functional differences between location and species were explored using community diversity metrics rather than individual pathway abundances.

While species had a significant effect (PERMANOVA, F = 4.81, P > 0.001) on functional community diversity and accounted for 41% of the variation present (R^2), location did not have a significant effect (P > 0.05, Table 3). As there was not a significant effect of location, samples from both locations were combined to determine how community functional diversity differed between species When pairwise comparisons were run between insect species (*P. grandis* not tested, N = 2), all had significantly different functional communities (PERMANOVA, P < 0.05, Table S7).

Discussion

In this study, we examined how the internal microbiota of aquatic macroinvertebrates differed among species belonging to different functional feeding groups in two Alpine stream habitats with different riparian conditions. While macroinvertebrate-microbe interactions have long been recognized as an essential component of understanding food web interactions in aquatic systems (Cummins & Klug 1979; Kaufman et al., 2000), this study represents the first comparisons among the internal microbial communities of macroinvertebrate species using high throughput genomic sequencing in Alpine stream communities. Two other recent studies using high throughput sequencing to compare the microbiota of aquatic macroinvertebrate functional groups occurred in Midwest streams (USA) (Ayayee et al., 2018), and associated with salmon decomposition (Pechal & Benbow 2016) in Alaska (USA).

While macroinvertebrate feeding groups differ in their predominant method of feeding, most aquatic species are omnivorous and readily uptake food from a variety of sources (e.g., scrapers ingesting particulate organic matter or eukaryotes during feeding on surfaces) though their ability to digest certain foods can vary due to multiple factors, including differences in their internal

322 microbiota (Pechal and Benbow 2016), pH, and oxygen conditions (Cummins and Klug 1979).
323 For example, it has been shown that crane flies (shredder, Diptera: Tipulidae) require microbial
324 conditioning of leaf surfaces for development and use gut bacteria to help break down ingested
325 food (Klug & Kotarski 1980; Lawson & Klug 1989). While in terrestrial systems, the functional
326 roles of microbes are widely documented (e.g., nitrogen fixation or cellulose degradation) (Alonso-
327 Pernas et al., 2017; Alonso-Pernas et al., 2018; Ayayee et al., 2018; Gupta et al., 2012), in aquatic
328 systems comparatively little research exists, but it is hypothesized that there are similar
329 relationships (Ayayee et al., 2018). Although we chose not to examine individual functional
330 pathways due to limitations of using gene amplicon data for this purpose in understudied systems
331 (Langille 2018; Langille et al., 2013; Radivojac et al., 2013), we found that the internal bacterial
332 functional diversity was distinctly different between species, with species explaining close to half
333 (41%) of the variation present. As macroinvertebrates from different feeding groups ingest and
334 process predominantly distinct forms of organic matter (Ayayee et al., 2018; Cummins & Klug
335 1979), it would be expected that their gut community assemblages would be adapted to different
336 functional roles, similar to terrestrial insects (Larsen et al., 2016; Mason et al., 2016). While further
337 research will be needed to elucidate exactly what functional roles these bacteria play, we
338 demonstrated both structural and functional differences among microbial communities of
339 macroinvertebrate species with different feeding behaviors.
340 We observed several differentially abundant bacterial taxa and were able to successfully
341 classify internal communities to both species and site with a high degree of accuracy. At all
342 taxonomic levels (phylum, family, and genus) there were significantly different abundances
343 between species, but no significant effects of location. In contrast to previous studies (Ayayee et
344 al., 2018; Pechal & Benbow 2016), which observed that predatory feeding groups had lower

phylogenetic diversity than grazers/scrapers and filterers, we observed that the predatory *D. alpinus* had higher diversity than *E. Alpicola* (scraper), regardless of location. Predators (Perlodidae: *D. alpinus*) showed similar levels of bacterial diversity (Shannon and Faith's PD) to species belonging to both filter (Hydropsychidae: *Hydropsyche* sp.) and shredder (Tipulidae: *T. maxima*) groups while species ~~of~~ belonging to the scraper groups (Heptageniidae: *E. alpicola*) had lower diversity than all others (then others?). The lower phylogenetic diversity of *E. alpicola* microbiota at the upstream prairie site, may be related to the lower diversity of benthic microorganisms that has been shown in the upper sections of Alpine rivers compared with downstream reaches (Falasco & Bona 2011). These results highlight that additional studies are needed to identify factors that shape macroinvertebrate gut microbial communities and how they relate to ecosystem function in Alpine stream habitats.

While we expected the diversity of microbial communities within *T. maxima* (shredder) to be higher at the forested site due to the presence of more heterogenous allochthonous and autochthonous resources below Alpine tree lines (Wilhelm et al., 2015), we observed no significant differences in alpha diversity or taxa composition (phylum, family or genus level), suggesting the shredder species we sampled at these sites may be acquiring gut communities with limited colonization from microbes associated with their food. Though location significantly impacted the beta diversity of the taxonomic bacterial communities, it had a smaller effect than species. When comparing bacterial community function, location no longer had significant impacts on diversity, suggesting that although taxonomic differences exist between sites, the taxa present may be playing similar functional roles within the insect guts, analogous to functional redundancy in other systems (Rosenfeld 2002).

Although differences in beta diversity were observed between sites, there were surprisingly no other significant taxonomic or functional differences between site for either *E. alpicola* (scraper) or *T. maxima* (shredder). As solar radiation and the presence of riparian vegetation significantly alters the taxonomic composition and function of biofilm communities (Wagner et al., 2015; Wilhelm et al., 2015), it would be expected to see differences between species of scrapers at the two sites that use biofilms as a predominant food source, if their microbiota simply reflected their diet. That no differences in taxonomic composition or functional diversity were observed between our scraper or shredder species at the two sites suggests their gut microbiota may be a result of selective colonization by taxa and not simply a reflection of their food. This similarity of microbial communities within insect species from different locations has previously been reported and may be a result of similar nutritional components and microbial species sorting due to similarities in the gut environment (e.g. morphology, pH, and oxygen conditions) (Anderson & Cargill 1987; Ayayee et al., 2018; Pechal & Benbow 2016).

Conclusions

Under the theoretical framework of the River Continuum Concept (RCC) and its derivatives (Junk et al., 1989; Vannote et al., 1980; Ward & Stanford 1995) high gradient, low order mountain streams provide a unique opportunity to investigate the impact of watershed conditions on microbial community assembly within macroinvertebrate species. While the tree line represents a drastic change in riparian conditions, we observed limited effects on internal microbial community structure and function, compared to the effects of macroinvertebrate species from different feeding groups. While there were site-specific differences in taxonomic diversity, these changes were not reflected in community function suggesting although different communities are present at the two locations, they provide similar functions. The observed dissimilarities between species ~~of~~

belonging to different functional feeding groups, regardless of riparian conditions, agrees with previous research in suggesting that conditions within their digestive system allows for selective colonization of microbes, with distinct functional roles, and do not simply reflect their environment/diet (Ayayee et al., 2018). That large differences in CPOM inputs (e.g., leaf material) and light conditions did not lead to differences in individual taxa or functional changes was surprising, particularly for our shredder and scraper species as leaf material and autotrophic organisms represent their predominant food source. As algal and fungal communities comprise important roles in primary production and organic matter processing in stream systems (Cummins 1974; Danger et al., 2013; Kuehn 2016) how these taxa differ between species and functional feeding guilds will require further investigation. While this study provides initial data on how species and habitat may be linked with gut bacterial communities in alpine systems, additional studies are needed to expand on this evidence and test multiple species of each functional feeding group in several catchments. Our study was limited to one species for each functional group (except for predators), making the distinction between the effect of species and functional group difficult to untangle. Larger, more comprehensive surveys and manipulation experiments are warranted to differentiate species and functional feeding group gut microbial community structure.

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Figure Captions

Fig. 1 a) Representative photo of tree line in Ostana. b) Study drainage basin in Italy showing the elevation/altitude for the higher elevation Alpine prairie location (Pian della Regina) and lower elevation forest location (Ostana). Altitude is displayed in meters above sea level.

Fig. 2 Photos showing the riparian vegetation cover of a) high elevation Alpine prairie station (Pian della Regina) and b) lower elevation forested station (Ostana).

Fig. 3 Internal bacterial communities of macroinvertebrate species: a) Differences in mass (mg) for *E. alpicola* and *T. maxima* at the two sites. Significance was determined by t-tests; b) Phylum level bacterial relative abundance between species; c) Differences in phylum level relative bacterial abundance between *E. alpicola and T. maxima*. Samples from the two sites were combined and only phyla with a relative abundance greater than 1% of the total relative abundance are shown. Samples were compared with Kruskal-Wallis tests with FDR correction; d) Differences in family level relative bacterial abundance between *E. alpicola* and *T. maxima*. Samples from the two locations were combined and only families which made up greater than

3% of the total relative abundance are shown, for lower abundance families see Figure S2. Significance between species were compared with Kruskal-Wallis tests and FDR correction. Error bars are SEM.

Fig. 4 Top ten genus level predictors (determined by mean decrease GINI) for a random forest model predicting species (*P. grandis* was not included in the model, N = 2). Error bars are SEM. Significance between pairwise comparisons (Mann-Whitney, FDR correction) are denoted by lowercase letters

Fig. 5 Differences in community diversity among macroinvertebrate species a) Differences in Faith's phylogenetic diversity (Faith's PD) between species. Comparisons between species were tested with Kruskal-Wallis tests with pairwise significance (Mann-Whitney) denoted by lowercase letters; b) Shannon diversity differences among species; c) PCoA plot (Jaccard distance) showing community differences due to taxonomic composition; d) PCoA plot (Jaccard) showing predicted functional differences in beta diversity among species. Ellipses represent 95% CI for the mean of each group. Legend is given in 5C.

Table captions

Table 1. Macroinvertebrate samples used for microbial sequencing.

458 **Table 2.** Differences in taxonomic beta diversity (Jaccard, PERANOVA) among
459 macroinvertebrate species and location (*P. grandis* not included, N = 2). SS = Sums of Squares,
460 MS= Mean Squares
461
462 **Table 3.** Differences in predicted functional beta diversity (Jaccard, PERMANOVA) among
463 macroinvertebrate species and location (*P. grandis* not included, N = 2). SS = Sums of Squares,
464 MS= Mean Squares
465

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