Dear Dr. Beckerman,

Thank you for editing our manuscript and providing us with the opportunity to submit a revised version. We have addressed all of the comments and suggestions by the two reviewers, which you will find below in blue text. While the suggested revisions were all relatively minor, we feel that they added up to really improve the manuscript and we hope that you will now find the paper suitable for publication in Ecology and Evolution.

Associate Editor

Comments to the Author:
This MS has been reviewed by two referees and both they and I feel the MS has some exciting and interesting potential, but needs several types of changes to improve clarity and impact. As the referees points out, there are several aspects of the data that are unacknowledged (mortality rates) and several pieces of theory (species richness) that are not woven into the story and data effectively enough. Both referees offer several papers that should improve the scholarship, hone analyses and improve insight. The authors should take these constructive comments as an effective road-map and revise the MS. Dealing with their combination of comments should make for an excellent paper.

Reviewer: 1

Comments to the Author:
Han et al test the potential for a dilution effect (a negative relationship between community richness and disease risk) using tadpoles and the parasitic chytrid fungus, Batrachochytrium dendrobatidis (= Bd) using laboratory microcosms. They conducted an ancillary experiment examining intra- and interspecific Bd transmission utilizing Anaxyrus as the host. Their results are largely confirmatory, especially within the Bd literature. Most notably, their main finding is quite similar to those of two recently published papers (Searle et al and Venesky et al, both of which are cited in the present manuscript), which found that general species richness and the specific identity of heterospecifics can redue the Bd infection intensity on Bufo. Nonetheless, I think that the present manuscript could be published after careful revisions to the manuscript, including a change in focus to highlight the novelty of their findings. Although I have a positive outlook on the prospects of this paper, I do have some reservations that that authors must address:

The authors must provide more details on the mortality situation, how many/which dead tadpoles were omitted from the qPCR analyses, and follow up in the Discussion on how this could impact their results. One of my main concerns is the high % of mortality across the treatments. Bd is not generally known to cause high mortality in tadpoles (although it can). However, ignoring the Bd+ treatment, Anaxyrus mortality in the Bd- treatment exceeded 80% in all species combinations. This is a bit surprising and raises concern. Readers will certainly see this and will be expecting some type of discussion about this. Please address this in the Discussion.

Nearly all tadpoles that died during the experiment were also tested for Bd in the mouthparts. In general, when tadpoles die the soft body parts are consumed or decompose rather quickly,

but the keratinized mouthparts where infection is localized take much longer to disappear. We therefore checked our experimental tubs very frequently (at minimum they were checked daily, and usually multiple times per day) to record mortality and to collect the carcasses of dead tadpoles. Of course, there were some tadpoles that disappeared completely between checks (105 animals were missing from both Bd and control treatments, see Table below), which we attributed either to opportunistic cannibalism, or consumption of a recently deceased tadpole.

We, too, were surprised by the degree of mortality among treatments for *A. boreas*, and particularly surprised not to find a significant effect of Bd on mortality in this species. Instead, the highest mortality for *A. boreas* occurred when they were combined with *Rana cascadae*. This makes sense to us given our observations of how these two species interact. *Rana cascadae* can be much larger than *A. boreas*, and can often be seen nibbling tails and otherwise "bullying" smaller individuals (of any species, but particularly *A. boreas* and smaller *R. cascadae*) especially in situations where food is limited. Healthy tadpoles will typically swim away, and may be seen occupying different areas of experimental tubs from larger *R. cascadae*. But if a tadpole is moribund, opportunistic cannibalism can occur. Importantly, the subset of dead tadpoles that went missing during the experiment was distributed evenly between Bd treatments, so the dilution effect we observed is unlikely to be artificially driven by tadpoles missing disproportionately from Bd treatments (see Table S2 below, which we have now added as supplementary information).

We have added more explicit discussion to the manuscript (lines 306-309) articulating that the nature of the interactions between species seems to be a more important driver of mortality dynamics in these communities than Bd infection, which might be especially important to consider since, as the reviewer notes, Bd has not been observed to cause precipitous mortality at the tadpole stage.

Table S2. The tadpoles that went missing during the experimental period, which were a small subset of the total number of dead tadpoles observed during the study (N=648).

MISSING TADPOLES

Focal spp		B		P		R		Totals
Bd treatmt		Bd+	Bd-	Bd+	Bd-	Bd+	Bd-	
Spp Combo	B	0	1	0	0	0	0	1
	BR	10	11	0	0	2	5	28
	P	0	0	4	0	0	0	4
	PB	0	1	1	0	0	0	2
	PBR	8	5	5	4	0	2	24
	PR	0	0	10	9	2	3	24
	R	0	0	0	0	9	13	22
Totals		18	18	20	13	13	23	105

Related to this, on lines 122-23, you need to be explicit here: was qPCR conducted on the dead tadpoles and included in your statistical models?

> We have clarified the wording here to show that qPCR was indeed conducted on all of the tadpoles (those that died during the experiment and those that were euthanized at the end of the experiment).

From the supplement, you stated that you ran qPCR on 324 tadpoles; I'm getting 336 tadpoles in the Bd+ treatment. Did you run 12 fewer qPCR samples because you were unable to recover these dead individuals? This is important and has implications for understanding your results. If the Anaxyrus with the highest loads died soonest (which is somewhat consistent with your results on 157-58) and if they were also the ones missing from your qPCR analysis, you could be creating a dilution effect by omitting the animals with the highest loads.

> We ran qPCR on 324 Bd+ and 324 Bd- tadpoles. Going back to the raw data, we recall that 4 tubs were omitted from the experiment (2 Bd+ and 2 Control treatment tubs) at the very start because the animals (*Pseudacris regilla*) that would have comprised those replicates were not adequately size matched (i.e., they were either much smaller or much larger than the other tadpoles). *Pseudacris regilla* often display a wide range of body sizes even when reared under identical laboratory conditions at low densities. In our experience, this size differential can affect agonistic relationships so we decided it was best to exclude these and did so in a balanced way by taking two tubs from each pathogen treatment (Bd+ and control).

The statistical analyses section (136-146) of the manuscript needs much more detail. For example, I'm not sure what you mean by "impacts of Bd" (line 141) and there is nothing in the supplement. Please be explicit as to how many models you ran and what the response and predictors were in each model. This ambiguity is annoying and confuses me even more as to what function the control treatment served. I'm assuming you ran some models with both Bd+ and Bd- treatments (perhaps for growth and development) but that you did not include the Bd- treatment in some models (= the infection severity model). Also, what statistical software did you use?

> We apologize for the confusion. We have rewritten this section for clarity (lines 166-188).

I think that the test for spillover is where this paper has its' novelty. I think that this paper would stand out more and have a larger contribution to the literature if the authors emphasized this experiment and the results therein. This is largely explored and has important implications, especially given the generality of their findings (Seare, Venesky, and Han have all found that Bufo carries high loads and can experience a dilution effect when other species are added to the mix). This might take a serious revision (which is why I recommended Major Revision and not Minor Revision) to frame this paper differently... but I think it will be worth it. I do, however, have 1 comment related to this specific experiment that I would like the authors to address in their revision. In the supplement, the authors show that Anaxyrus had pretty low Bd loads (~30 genome equivalents). Was this the Bd load at the start or completion of the 40 day transmission trial? If the start, I'd like to know that they were actually still infected by the end. I'm asking for

these data because this is a low infection load and one that could be cleared by a tadpole, which would mean that you weren't actually conducting a transmission trial.

> We found these results really interesting as well. As suggested, we have restructured the paper in several places to highlight the results and implications of the spillover experiment (see specifically all five points of the Abstract, line 90 of the Introduction; the Methods for the transmission experiment have now been moved from Supplementary Materials to the main text, lines 190-201; Results for the transmission experiment are summarized in lines 251-253).
>
> The Bd loads reported were from the end of the experiment (now clarified in line 251).

I hope that the authors consider these comments and that they are willing to take on the revision.

Reviewer: 2

Comments to the Author:
The authors empirically tested the relationship between species richness and infection loads in three sympatric amphibian species in a lab arena. A dilution effect was found to occur when considering one of the amphibian species as a focal species, but not in relation to species richness. The paper is well written and represents an important contribution to this literature. I have only minor comments.

Whilst the value of a tractable system is very clear I would like to see more attempt by the authors to relate the findings to free-living conditions. Given, for example, the inter-specific competition and predation in multi-species communities what is the likelihood the dilution effect will be of 'benefit' to Anaxyrus? What are the field assemblages of these species? Are infection loads under natural conditions known? How much does your experiment mirror actual field conditions?

> These are great suggestions. These species occur frequently in the field together and predominate the montane communities of the Oregon Cascades and have also been found infected in the field (lines 118-121). We agree with the reviewer – whether the dilution effect we observed for *A. boreas* benefits them somehow in natural communities will depend heavily on how 'important' Bd is compared to interspecific interactions such as competition and predation. In our experiments, Bd seemed to be less 'important' than competition. We did see that *Anaxyrus* in mixed species settings had much reduced Bd infections, but they had greater mortality rates, probably because they were outcompeted by the other two species (further evidenced by the increases in mass and length for these other species when combined with *Anaxyrus*) (discussed from line 302). We add more explicit discussion on this point in lines 305-308, and lines 327-330.

General comment – Given recent reviews by Salkeld et al (2013) and Wood and Lafferty (2013)

I would argue that one would not necessarily expect a dilution effect to occur as a function of species richness (line 33), but as suggested by the authors in their very nice overview of the subject in the introduction, the dilution effect is more nuanced than a simple species richness – pathogen reduction relationship. Much work (by Ostfeld and co-workers for example) has focused on the rather precise host-parasite conditions required for the dilution effect to 'work' in a given system - reservoir competence, relative abundance etc.

> Yes, we definitely agree. I see that the connotation of that sentence in the Abstract gives the impression of a more dogmatic expectation between dilution and species richness, so we reworded point number four, and deleted the clause that dilution did not occur as a general function of species richness.

Line 37 – make it clear that 'identity' refers to species and not individual identity (which was not measured), which is how I interpreted this until I had read the manuscript in full.

> We now clarify that we mean "species identity" in the first mention (in point 5 of the Abstract) and throughout the manuscript.

Methods - More info is needed about the inocula. Is this very high/low? What was the rationale for the dose choice?

> We harvested Bd from agar plates that were cultured specifically for this experiment, so our infectious dose was determine mostly by how well the fungus grew on these plates in the month leading up to the experiment. The reviewer raises a really good point here – there isn't any notable standardization of Bd inoculation doses across experimental studies. This may be due in part to the difficulty in quantifying a realistic infectious dose in nature as Bd can be shed from a number of living (other frogs) and non-living (snake skin, other keratinized matter) sources. A quick search of some recent experimental inoculation studies revealed infectious doses ranging from 10^4 to 10^7 zoospores, but these zoospore doses were added to individual frogs (Greenspan *et al.* 2012), or represent the zoospores dose added to a 10-gallon aquarium containing numerous tadpoles (Searle *et al.* 2011) or zoospores per liter of water which was then aliquoted across beakers containing 200 mL of water and single tadpoles (Romansic *et al.* 2011), representing a very wide range of dose concentrations. We added 10^8 zoospores to each tub, which holds approximately 2 L of water. Based on the literature, the dose we used seems rather unremarkable (not extraordinarily high or low).

Line 196 – Is dilution really measured here or are you measuring differential susceptibility? Are you defining dilution as differential susceptibility?

> This is a very interesting point. It is entirely possible that the dilution result which we report for *A. boreas* could be driven by intrinsic differences in susceptibility under different species combinations, as opposed to the more direct removal of zoospores by other species as we postulate in the Discussion. In other words, each of the 6 toad tadpoles that we inoculated with Bd in the single species treatment are less susceptible to

infection at the individual level compared to each of the 2 toad tadpoles we inoculated with Bd in the mixed species treatment. It seems like an experiment to test for this would need to control for the possible removal of zoospores via filter feeding in order to make conclusions about intrinsic differences in susceptibility within different host communities. We didn't control for this in our study, but it seems like an important hypothesis for future exploration.

REFERENCES

Greenspan, S.E., Calhoun, A.J., Longcore, J.E. & Levy, M.G. (2012) Transmission of Batrachochytrium dendrobatidis to wood frogs (Lithobates sylvaticus) via a bullfrog (L. catesbeianus) vector. *Journal of wildlife diseases*, **48**, 575–582.

Romansic, J.M., Johnson, P.T.., Searle, C.L., Johnson, J.E., Tunstall, T.S., Han, B.A., Rohr, J.R. & Blaustein, A.R. (2011) Individual and combined effects of multiple pathogens on Pacific treefrogs. *Oecologia*, 1–13.

Searle, C.L., Biga, L.M., Spatafora, J.W. & Blaustein, A.R. (2011) A Dilution Effect in the Emerging Amphibian Pathogen Batrachochytrium Dendrobatidis. *Proceedings of the National Academy of Sciences*, **108**, 16322–16326.