Reviewer Comments for the Manuscript

We appreciate the careful reading and comments of all three referees. Below, we copy their comments (black) and answer to their questions and criticisms (blue)

Reviewer #1

Suitability Assessment

Suitable Quality? No

Sufficient General Interest? No

Conclusions Justified? Yes

Clearly Written? Yes

Procedures Described? Yes

Supplemental Material Warranted? Yes

Sufficient data/samples? Yes

Comments

The manuscript by Villalobos et al. reports an experimental study of the diffusion dynamics of a passive tracer in a liquid droplet containing swimming bacteria. The main findings as stated in the abstract and in the conclusions are:

1. "We demonstrate that momentum transfer from the bath to the tracer can be effectively described as colored noise, characterized by temporal memory and an enhanced effective diffusivity significantly larger compared to thermal Brownian motion values."
2. "Using a stochastic analytical framework, we extract the temporal memory and diffusivity parameters that define such an active bath."
3. "Notably, the diffusivity scales linearly with bacterial concentration, modulated by a factor representing the role of confinement expressed as the ratio of the confining radius to the probe radius."

The first is now a well-established result, which has been explored in many details and consequences in a long series of papers following Ref. [2] in the manuscript.

The proposed "stochastic analytical framework" is a quite standard Langevin simulation of a confined overdamped particle subject to coloured noise (OUP). The same model has already been described by some of the authors in ref. [45] and used here to fit experimental MSD to extract the statistical parameters of the fluctuating force.

That diffusivity in active baths scales linearly with bacterial concentration was also established for bulk suspensions. In ref. [5] for example it is found that diffusivity is linear with the product of bacterial density and mean speed. Here, the authors find a good correlation (not exactly a "collapse") between the active diffusivity Db and the product of bacterial density and a confinement parameter which is positively correlated to bath speed and particle mobility.

In conclusion, I find no major surprises, as the overall findings align well with bulk results and expected confinement effects. In addition, both the experimental and theoretical methods have already been published by some of the authors. While the present study of tracer dynamics in confinement is an interesting exercise, it appears to be more of an incremental contribution, making it better suited for a specialized journal.

Indeed, the increase in diffusivity of *tracer particles* with the concentration of microswimmers due to the non-thermal agitation produced by the active bath is a widely known experimental fact. However, please note that, from our analysis, the bath speed u_b, persistence time τ_b and diffusivity $D_b = u_b^2\tau_b$ are properties of the *bath* itself and not of the tracer. We have already discussed this in our previous paper Phys. Rev. E 110, 014610 (2024) (Ref. [45] in our manuscript), were we presented a protocol to extract the properties of an active bath from the dynamics of a tracer particle. One of the key points to retain there is that there is not thermalization due to the temporal persistence of the noise, which causes the properties of the particle's dynamics (diffusivity, speed and persistence time) to be related, but not equal, to the active bath properties. Thus, although the increase of particle diffusivity is known to increase linearly with the "active flux" (Phys. Rev. Lett. 106, 048102 (2011)), our conclusions for the increase of bath diffusivity with bacterial concentration is subtly but fundamentally different.

We agree that the description of the active bath in terms of a colored Orstein-Uhlenbeck (OU) noise is not new and has been proposed before as a model to describe this system. Despite the above, it is important to note that the OU model has not been fully validated, thus our experiment provide additional, but still partial, validation.

Having said all that, the third point mentioned by the referee, and more particularly, the role of confinement in the properties of the active bath, is the main novelty of our work. To our knowledge, the influence of confinement in the properties of an active bath has not been reported previously. We realize from the comments of all three referees, that we did not efficiently present this message, and therefore we have rewritten the abstract and significant statement, as well as some sections of the manuscript. We hope that these changes contribute to most clearly stating the novelty of our work. [MLC: Pending: Do the changes!]

Below some comments which I hope will be useful to the authors:

(a) I feel that the paper concentrates too much on a bulk analysis and misses the opportunity to explore the more peculiar features of the confinement. For example, it would be interesting to see a spatial map of the probability density of the tracer and compare it with the predicted stationary state of OUP particles in an external potential. Is there any accumulation on the boundary and how does it depend on bath properties?

We have included some representative examples of the probability density of tracer position, experimentally determined for some relevant 3D tracks ($P(\tilde{r})$) and for some representative examples of the 2D tracks ($P(\tilde{\rho})$), see new supplementary figures S3 and S4.

Say something about $p(r)$.

The $P(\tilde{\rho})$ data agree reasonably well with the distributions predicted for an OU particle in a harmonic potential, although the agreement degrades when the particle explores a large region of the confinement (large values of $\langle \rho^2 \rangle$).

These data contribute to further validate the OU model for the active bath. Also, they show that, due to buoyancy, the particles spend a large proportion of time close to the spherical boundary.

PIV and flux theory. Focus on the dependence on concentration and confinement. See the OLD draft for such an attempt.

(b) I do not understand why in paragraph "Friction coefficient" the authors suggest that "friction enhancement can be viewed as an effective lubrication effect arising when the tracer particles are in close proximity to the confining walls". We know that at low Reynolds numbers hydrodynamic interactions with walls can be quite long ranged (1 over wall separation). I think that a better discussion of what is known about viscous drag in spherical confinement should be included.

Develop the friction theory further. Discuss Aponte-Rivera, Physical Review Fluids 1.2 (2016): 023301.

(c) Since the authors have access to the bacterial flow field v inside the droplet, it would be interesting to discuss, for example, how the average $\langle v^2 \rangle$ depends on the droplet size, and perhaps use $\langle v^2 \rangle$ to look for a better scaling formula for Db, e.g. replacing the dependence on confinement radius.

Make v^2 vs. D_d plots.

(d) Is the presence of Brownian motion also responsible for the short time exponent of MSD being smaller than 1?

For the range of tracer particle radius used in the experiments, $R_i \in [2, 22]$ microm, in an aqueous environment at room temperature, the typical (Brownian) diffusion is expected to be several orders of magnitude lower than the measured MSDs. As an example, the MSD due to Brownian motion was plotted in Fig. S7. Thus, we expect no influence of Brownian motion in the results.

[MLC: Add MSD due to Brownian diffusion to Fig. S7.]

Reviewer #2

Suitability Assessment

Suitable Quality? No

Sufficient General Interest? No

Conclusions Justified? Yes

Clearly Written? Yes

Procedures Described? Yes

Supplemental Material Warranted? Yes

Sufficient data/samples? Yes

Comments on the Significance Statement

My criticism of the significance statement is similar to that of the abstract: Rather general statements about the different properties of active and thermal baths.

Comments

This manuscript describes results of a combined experimental and theoretical study of buoyant passive tracers in an active bath of motile bacteria confined in a droplet. It is shown that the effect of the bath on the tracer can be effectively described as coloured noise. The effective diffusivity is found to be significantly larger than for thermal Brownian motion. The diffusivity scales linearly with bacterial concentration, modulated by a factor related to confinement, as expressed by the ratio of confining and probe radius.

The different properties of active compared to thermal baths have found considerable attention recently, both experimentally and theoretically – and many interesting behaviours have been discovered. On the theoretical side, an effective description of the bath by coloured noise has emerged.

To my knowledge, the effect of a confinement in a droplet has never been investigated. However, to a considerable extent, the results of the current study seem to agree well with established results of previous investigations. As far as I can see, the most unexpected behaviour is the increase of the "bath" velocity with increasing bacterial density (Fig. 3b), and the dependence of the drag coefficient on the droplet radius (Fig. 3c). Here, it should be noticed that the "bath" velocity is in fact an effective particle velocity. As more bacteria collide with the particles at higher density, an increasing particle velocity seems not to be too surprising. The reason for the dependence of the drag coefficient on the droplet radius is not obvious, but is not investigated further.

As stated by the referee, the effects of confinement in the properties of an active bath have not been studied before, and this corresponds to the main contribution of our work. We realize from the comments of all three referees, that we did not efficiently present this message, and therefore we have rewritten the abstract and significant statement, as well as some sections of the manuscript. We hope that these changes contribute to most clearly stating the novelty of our work.

Please note that the bath parameters u_b, τ_b and D_b are indeed parameters of the bath and not of the tracer. These parameters characterize the colored noise ($\mathbf{u}$ in Eq. [1]) and thus describe the fluctuating forcing of the bacterial bath acting on the tracer. As we have already proven (see Phys. Rev E 110, 014610 (2024)), the particle speed is not equal to the bath speed due to the temporal persistence of the noise ($\tau_b \neq 0$). The whole point of our analysis is to extract the bath properties from the dynamics of the tracer.

[MLC: Comment of $u_b = u_b(n)$ which for me is to be expected, but in fact is new.] [MLC: Comment on confinement effect on friction.]

Some comments and questions:

(1) **Abstract:** The first have of the abstract reads like an introduction to non-equilibrium statistical physics. I suggest to focus more on the results of the current study.

(2) **Introduction:** Some related references:

- Hydrodynamics of polymers in an active bath
  A. Martin-Gomez, T. Eisenstecken, G. Gompper, and R.G. Winkler,
  Phys. Rev. E **101**, 052612 (2020)
- M. S. Aporvari, M. Utkur, E. Ulku Saritas, G. Volpe, and J. Stenhammar,
  Anisotropic Dynamics of a Self-Assembled Colloidal Chain in an Active Bath,
  Soft Matter **16**, 5609 (2020)
- Low efficiency of Janus microswimmers as hydrodynamic mixers
  M.R. Bailey, D.A. Fedosov, F. Paratore, F. Grillo, G. Gompper, L. Isa,
  Phys. Rev. E **110** (4), 044601i (2024)
- Robust Edge Flows in Swarming Bacterial Colonies
  He Li, Hugues Chaté, Masaki Sano, Xia-qing Shi, and H. P. Zhang,
  Phys. Rev. X **14**, 041006 (2024)

(3) What is the dependence of the sedimentation velocity (or effective drag coefficient of a particle in a droplet on the droplet radius in a passive system? [MLC: Aponte-Rivera]

This is a careful and detailed study, but in my opinion does not contain sufficient significantly new results to justify publication in an interdisciplinary journal such as PNAS.

Reviewer #3

Suitability Assessment

Suitable Quality? Yes

Sufficient General Interest? Yes

Conclusions Justified? Yes

Clearly Written? No

Procedures Described? No

Supplemental Material Warranted? No

Sufficient data/samples? No

Comments

This is a very interesting, experiment-based study of passive tracers in droplets containing a passive liquid and actively-moving bacteria. Together with stochastic simulations and some analytical calculations, the authors present important findings elucidating the dynamics of this mixture under biologically relevant confinement.

While I find this study to be very timely and relevant, a number of details are missing. In particular more information could be included in the SM. The authors should conretely respond to the points below.

Is this paper PNAS material? The topic is certainly worthy of publication on the level of PNAS. Currently, the presented material and discussion are more on the level of PNAS Nexus, but I am happy to reconsider my opinion after revision by the authors.

(1) From what I can tell the authors show ensemble-averaged data for the MSD. I am wondering whether they could show individual MSD data evaluated through time averages. The necessarly occuring amplitude scatter could give interesting clues on the system, see, e.g., Fig 1 in Phys Rev Lett 132, 088301 (2024). Moreover, it could be insightful to analyse the Ergodicity Breaking parameter, the variance of the amplitude scatter of the time-averaged MSD curves, as function of the measurement time, see, e.g., PCCP 16, 24128 (2014). Does this correspond to extected behavior in an OU-dynamics?

In general, some more detailed information on how the MSD is determined from data (e.g., number of trajectories) should be provided in the SM.

In fact, all of our MSDs were computed as time-averaged MSD for single tracers. We emphasize this in the new version of the paper. Time-averaged MSD obtained for single particle tracking experiments aligns with our objective, which was to extract the bath parameters for individual realizations of encapsulated bacterial suspensions. Given that in each experiment could vary, independently, the bacterial concentration n, the confinement radius R_o and the tracer radius R_i, ensemble averages are meaningless and indeed large amplitude variations occur.

(2) Is there a chance to reconstruct the probability density function for the tracer particle? From computational studies of long-range dependent motion it is known that interesting enrichment or depletion occurs close to boundaries, see, e.g., New J Phys 21, 022002 (2019). It would be nice to see whether similar effects occur in this finite-persistence scenario.

We present now some examples of the probability density of tracer position, experimentally determined for some of the 2D tracks in the (x, y) plane $(P(\tilde{\rho}))$ and for some of the 3D tracks $(P(\tilde{r}))$. These PDF agree reasonably well with the distributions predicted for an OUP tracer particle in a harmonic potential. The data, thus, does not provide any evidence of increased or decreased accumulation of the tracer on the confinement surface. [MLC: Cristian is preparing the plots.]

Moreover, I have a question about the shown trajectories in Fig 1c. The tracer has a finite radius, yet the (presumabely center-of-mass) data come very close to the drawn circle: does the tracer locally protrude out of the droplet? This point should be discussed, also with respect to boundary effects known from bacteria solutions close to hard boundaries.

We did not observe any physical protrusion of the tracer beads outside the droplet interface. The proximity of the plotted trajectories to the drawn droplet boundary (Fig. 1c) is due to the visual representation: trajectory points represent the center of mass position of the bead, which have a finite radius of 2 µm. The effective droplet radius used for analysis, $R = R_o - R_i$, explicitly accounts for this bead radius by subtracting it from the outer droplet radius measured at the equatorial plane.

(3) The simulations details should be provided explicitly. Moreover, sample trajectories from the simulations should be shown and compared with the recorded data. How do reflective (i.e., hard) boundary conditions really reflect the actual system?

[MLC: Rodrigo?]

(4) Can it be shown explicitly that the increments of the motion are stationary, as expected for OU-driven motion? Can the "lubrication-like" forces mentioned in the manuscript be related to some higher localisation probability next to the surface?

[MLC: In PCCP 16, 24128 (2014) they talk about testing stationarity] [MLC: For friction, Aponte-Rivera?] Each trajectory was recorded at 50 fps and lasted, typically, 5 min (for beads in the (x, y) plane) or 10 min (for double emulsions in the (x, y) plane), thus comprising between 15000 and 30000 data points. For the 3D tracking of beads and (x, z) tracking of double emulsions, the trajectories typically comprised less data points due to experimental difficulties in the tracking procedures. This means that, although the statistics was enough for obtaining smooth curves for MSD and satisfactory fits, it was not enough for an analysis of stationarity. As noted by Referees #1 and #2, the OU model for an active bath has been proposed previously by a number of authors (see, for example, Refs. [28, 29, ??]), as was taken here as a working hypothesis that could not be further validated.

[RS: Decir que las trays son cortas y no podemos validar todas las hipótesis del modelo. Usamos el modelo para describir al baño. Es nuestra hipótesis de trabajo. Por otro lado, los otros referees dicen que el modelo está validado. Podemos decir que nosotros avanzamos a la validación del modelo]

Minor Points

(1) In Fig S1 please choose a different color coding for the velocity scale, it is almost invisible for readers with red-green vision impairment.

We have changed the color map.

(2) Ref 1: note that nouns in the German title should be capitalized: "Theorie", "Wärme", "Bewegung", "Flüssigkeiten", and "Teilchen".

Corrected.