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Variability and host density independence in inductions-based estimates of environmental lysogeny

Ben Knowles[1*†], Barbara Bailey[2], Lance Boling[1], Mya Breitbart[3], Ana Cobián-Güemes[1], Javier del Campo[4], Rob Edwards[5,6,7], Ben Felts[2], Juris Grasis[1], Andreas F. Haas[1], Parag Katira[8], Linda Wegley Kelly[1], Antoni Luque[2,5,7], Jim Nulton[2], Lauren Paul[1], Gregory Peters[1], Nate Robinett[1], Stuart Sandin[9], Anca Segall[1,7], Cynthia Silveira[1], Merry Youle[10] and Forest Rohwer[1,7*]

Temperate bacterial viruses (phages) may enter a symbiosis with their host cell, forming a unit called a lysogen. Infection and viral replication are disassociated in lysogens until an induction event such as DNA damage occurs, triggering viral-mediated lysis. The lysogen–lytic viral reproduction switch is central to viral ecology, with diverse ecosystem impacts. It has been argued that lysogeny is favoured in phages at low host densities. This paradigm is based on the fraction of chemically inducible cells (FCIC) lysogeny proxy determined using DNA-damaging mitomycin C inductions. Contrary to the established paradigm, a survey of 39 inductions publications found FCIC to be highly variable and pervasively insensitive to bacterial host density at global, within-environment and within-study levels. Attempts to determine the source(s) of variability highlighted the inherent complications in using the FCIC proxy in mixed communities, including dissociation between rates of lysogeny and FCIC values. Ultimately, FCIC studies do not provide robust measures of lysogeny or consistent evidence of either positive or negative host density dependence to the lytic–lysogenic switch. Other metrics are therefore needed to understand the drivers of the lytic–lysogenic decision in viral communities and to test models of the host density-dependent viral lytic–lysogenic switch.

Lysogenic dynamics can disassociate viral infection and production, leading to virus–host predator–prey feedbacks, densities and ecosystem impacts divergent from those predicted under lytic dynamics[1–3]. Although the majority of cultured laboratory and environmental bacterial strains are known lysogens[4,5], quantifying the fraction of lysogens in natural mixed communities remains challenging. The prevalence of lysogeny is most commonly estimated by using the DNA-damaging agent mitomycin C to induce prophages (viruses that have established sustained intra- or extra-chromosomal residence in their hosts) to enter the lytic cycle and produce quantifiable viral progeny[6,7]. Lysogeny has been diagnosed using this technique in laboratory strains for half a century[6,8]. However, treatment of lysogens can yield induction, unsuccessful induction, or inhibition of host and viral production under different mitomycin C concentrations (Table 1)[6] that vary on a strain-specific basis[9]. Inductions are difficult to interpret, even under single-strain laboratory conditions. Despite these challenges, in the 1990s Jiang and Paul[4,10] extended this induction method from laboratory strains to bacterial isolates from mixed natural communities, showing that lysogeny is a common viral strategy in the environment (25–62.5% of strains were lysogens). Higher percentages were commonly observed under oligotrophic conditions than in eutrophic systems, suggesting links between the rate of lysogeny, nutrient regime and host density[4,10].

Concurrent studies directly probed natural communities by adding mitomycin C into samples of sea water[11,12]. In those studies, the fraction of lysogenic cells (FLC, hereafter referred to as the fraction of chemically inducible cells, FCIC, due to disassociation of lysogeny and the induced fraction, as a percent of total cellular density) was estimated as

$$\%\ \mathrm{FCIC} = \frac{(V_\mathrm{i} - V_\mathrm{c})/B}{C} \times 100 \qquad (1)$$

using viral densities in the induced (V_i) and control (V_c) treatments, burst size B and host density C (cells per ml or g of sample) before incubation[11–13]. In contrast to earlier isolate-based studies, this mixed-community approach showed that either FCIC was insensitive to ecosystem nutrient status[12] or was higher under eutrophic conditions[11].

Subsequent research led to the consensus view that the frequency of lysogeny was inversely related to host density and nutrient availability[14–17]. This suggested that lysogeny provides a temporary refuge for viruses when hosts are starving and scarce[14], seemingly providing a low-density lysogenic dynamic complementary to the

[1]Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, California 92182, USA. [2]Department of Mathematics and Statistics, San Diego State University, 5500 Campanile Drive, San Diego, California 92182, USA. [3]College of Marine Science, University of South Florida, 140 Seventh Avenue South, St Petersburg, Florida 33701, USA. [4]Department of Botany, University of British Columbia, 3529-6270 University Boulevard, Vancouver, British Columbia V6T 1Z4, Canada. [5]Computational Science Research Center, San Diego State University, 5500 Campanile Drive, San Diego, California 92182, USA. [6]Department of Computer Science, San Diego State University, 5500 Campanile Drive, San Diego, California 92182, USA. [7]Viral Information Institute, San Diego State University, 5500 Campanile Drive, San Diego, California 92182, USA. [8]Department of Mechanical Engineering, San Diego State University, 5500 Campanile Drive, San Diego, California 92182, USA. [9]Scripps Institution of Oceanography, University of California San Diego, 950 Gilman Drive, California 92903, USA. [10]Rainbow Rock, Ocean View, Hawaii 96737, USA. [†]Present address: Department of Marine and Coastal Sciences, Rutgers University, 71 Dudley Road, New Brunswick, New Jersey 08901, USA. *e-mail: benjaminwilliamknowles@gmail.com; frohwer@gmail.com

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Table 1 | Phenotypes observed when mitomycin C is added to a lysogen lineage in correct, under- and over-dose concentrations.

Dose	Change in host densities (relative to control)	Change in viral densities (relative to control)	FCIC range	Conclusion	Error type
Correct dose	Decline (induction)	Increase (induction)	>0%	Lysogeny found at correct rate	None
Under-dose	No change (unsuccessful induction)	No change (unsuccessful induction)	0%	Lysogeny underestimated	Type II
Over-dose	Decline (inhibition)	Decline (inhibition)	<0%	Lysogeny underestimated	Type II

Under- and over-dosing leads to unsuccessful and inhibited induction, respectively. Although these different dosage categories yield distinctive FCIC ranges when lysogens are probed, incorrect dosage of either form consistently yields Type II error.

modelled high-density lytic 'kill-the-winner' dynamic[18]. By contrast, recent work using extensive field direct counts, experiments and viromes (c.f. chemical induction) suggests that lysogeny is favoured when hosts are highly abundant and rapidly growing[1]. We call this model 'piggyback-the-winner'. The association of high host densities with high rates of lysogeny reflects the benefits of lysogeny to hosts, including prophage-mediated immunity against further infection[19,20], protection from protist predation via virulence factors, and gain of metabolic functions[21–23].

Approximately 40 studies measuring rates of lysogeny in mixed natural communities using mitomycin C induction have been published in the twenty years since the pioneering work of Jiang and Paul[4,10] (Supplementary Table 1). We have compiled data from published environmental induction studies and found that FCIC is highly variable and seldom correlated with host density. Attempts to identify the source(s) of FCIC variability revealed issues with estimating the rates of lysogeny using mitomycin C induction. We propose that lysogeny and FCIC are not equivalent and that FCIC measurements obscure relationships between host density and lysogeny.

Results

Global distribution of FCIC studies. Clusters of saltwater studies in the Gulf of Mexico (18% of all studies) and predominantly freshwater studies in France (26%) account for 44% of all FCIC studies (Fig. 1a). Of the 39 studies, most (90%, $n = 35$) were from aquatic environments. Of the 352 published data points compiled, 91% (320 points) came from aquatic environments (compared to 32 points from sediment and soil combined).

Global host density versus published FCIC values. The full 352-point global data set was analysed for a correlation between host density and FCIC (Fig. 1b). This analysis showed high variability and no significant support for the established paradigm that FCIC is inversely related to host density ($P = 0.07$, $n = 352$, m (slope) $= -0.08$, $R^2 = 0.01$, 39 studies; linear regression with FCIC and host density log-transformed). Type II regression yielded similar results (95% confidence interval (CI) [−0.17, 0.01] for the slope, $m = -0.08$, $n = 352$, $R^2 = 0.01$).

Owing to the high variability between FCIC and host density, 468 further data points (that is, site averages) would be required to find any (positive or negative) significant global-level relationship between host density and FCIC (two-tailed power analysis, power = 0.8, $P < 0.05$; Fig. 1b). This represents a 2.3-fold increase in data over that generated in the two-decade history of the field.

Host density versus published FCIC values by environment. A high degree of variability and lack of significant relationships was also observed when FCIC was plotted against host density for specific environments (Fig. 2 and Supplementary Fig. 1). Regressions in freshwater, saltwater and sediment data sets showed no significant effect of host density on FCIC (Fig. 2; freshwater: $P = 0.16$, $n = 131$, $m = -0.18$, $R^2 = 0.02$, 15 studies; saltwater: $P = 0.76$, $n = 189$, $m = -0.02$, $R^2 < 0.01$, 22 studies; sediment: $P = 0.32$, $n = 19$, $m = -0.10$, $R^2 = 0.06$, 3 studies; linear regressions with FCIC and host density log-transformed). The only environment with a slope significantly different from zero was soil, where a positive relationship was observed ($P < 0.01$, $n = 13$, $m = 0.20$, $R^2 = 0.58$, 2 studies; linear regressions with FCIC and host density log-transformed). Global regression lines explained 58, 6, 2 and <1% of the variability between FCIC and host density in soil (2 studies), sediment (3 studies), freshwater (15 studies) and saltwater (22 studies), respectively (Fig. 2). Type II regressions confirm these results (freshwater: 95% CI [−0.42, 0.07] for the slope, $m = -0.17$, $n = 131$, $R^2 = 0.02$, 15 studies; saltwater: 95% CI [−0.14, 0.11] for the slope, $m = -0.02$, $n = 189$, $R^2 < 0.01$, 22 studies; sediment: 95% CI [−0.29, 0.10] for the slope, $m = -0.10$, $n = 19$, $R^2 = 0.06$, 3 studies; soil: 95% CI [0.09, 0.32] for the slope, $m = 0.20$, $n = 13$, $R^2 = 0.58$, 2 studies). Evaluation of within-study variability expressed as median within-study ranges in FCIC from each environment ranked sediment as least variable (3.9% median range), followed by freshwater (12.60% median range), seawater (26.62% median range) and soil (most variable, 36.55% median range) (Fig. 2).

Due to the high variability between FCIC and host density, 386, 15,450 and 110 further values (that is, site averages) are required to find any (positive or negative) significant relationships between FCIC and host density in freshwater, saltwater and sediment environments, respectively (two-tailed power analysis, power = 0.8, $P < 0.05$; no further values are required in soil) (Fig. 2). This represents approximately 4-fold, 83-fold and 7-fold increases in FCIC sampling over extant data accumulated in the past two decades in these environments.

Host density versus FCIC values within studies. Non-significant relationships ($P > 0.05$) were also the norm within studies (Fig. 3a, Supplementary Table 1 and Supplementary Fig. 1). Of the 42 analyses (39 studies, three of which include samples from two environments; Supplementary Table 1 and Fig. 3a), only five studies showed significant relationships (11.90% of studies, reported in refs 16, 17, 24 and 25 and the sediment subset of ref. 26). While negative relationships (slopes) between FCIC and host density are most common, 40% of freshwater, 32% of saltwater, 33% of sediment and both soil studies showed positive trends (linear regressions with FCIC and host density log-transformed; Figs 2 and 3a and Supplementary Fig. 1). Aquatic environments with more intensive sampling, either as individual data points or as number of studies, have lower R^2 values than the less-sampled sediment and soil environments (Supplementary Table 1 and Fig. 2).

Distribution of published FCIC values. The most common published FCIC values for global and freshwater, saltwater and sediment environments are 0–5% FCIC. Pooled published FCIC data show a truncated normal distribution centred approximately around 0% FCIC. Commonly excluded FCIC values ≤0, possibly generated by host inhibition via mitomycin C treatment (Table 1), probably fill out the lower range (Fig. 3b).

Frequency of FCIC values ≤0. To assess the frequency and distribution of FCIC values ≤0, FCIC was estimated in technical

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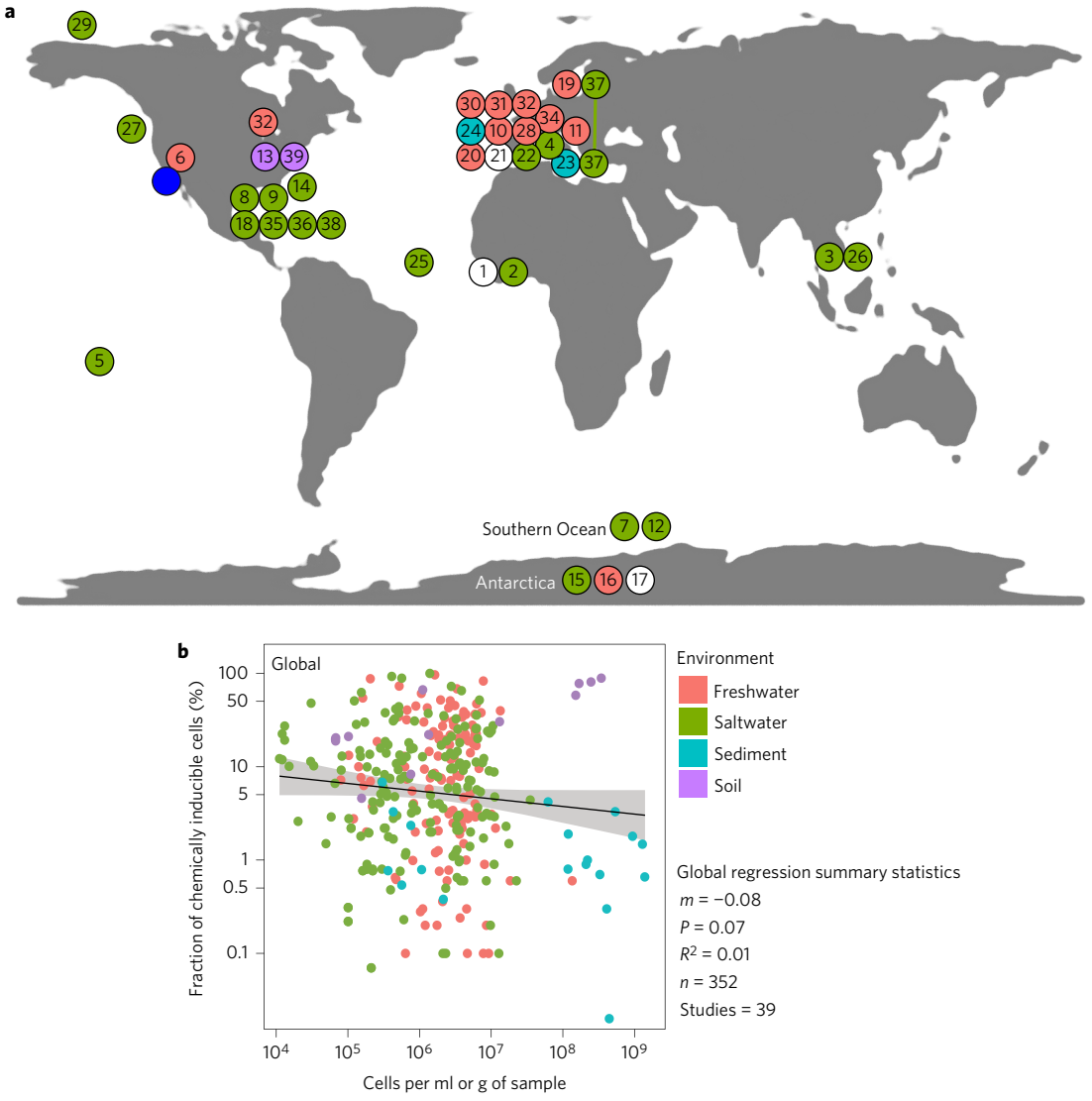

Figure 1 | Locations and global linear regression of data from meta-analysis of 39 published studies based on chemical induction of lysogens. **a**, Schematic map showing the global distribution of studies included in the meta-analysis, numbered by reference and coloured by environment (Supplementary Table 1). Note that samples for study 1 were from both freshwater and sediment, for studies 17 and 21 were from freshwater and saltwater (white circles), and for study 37 were from two distant marine environments. Blue circle with no number: freshwater and saltwater sampling sites for the current study around San Diego, California, USA. **b**, Global synthesis of FCIC data across host density across environments (both axes log-transformed). Black: global line of best fit. Grey shading: global 95% prediction interval. Summary statistics (m, P, R^2 and n) are from linear regression across all data. Map adapted from https://commons.wikimedia.org/wiki/File:BlankMap-World6.svg.

replicates from three saltwater and three freshwater sites. Highly variable FCIC values were observed within and between sites (Fig. 4a). The within-site average FCIC values varied from −2.17 to 9.61% and the within-site ranges were up to 14.51% (Supplementary Table 2 and Fig. 4a). The site with the highest variability, Spanish Landing, showed both negative and positive values (−4.17 to 10.34%; coefficient of variation of 1.44), despite a markedly consistent host density across replicates (5.09×10^6 to 6.55×10^6 cells per ml, coefficient of variation of 0.11; Fig. 4a). Four sites showed higher variability between technical replicates than was observed in the 25th percentile of published freshwater and saltwater environmental studies (Fig. 5c; comparison of ranges in each study or site). FCIC values ≤0 were observed in four of six sites and spanned the full range of host densities, indicating that 'unsuccessful' induction events or host inhibition are independent of host density and site (Fig. 4a).

Effects of excluding FCIC values ≤0. When all data points were considered, half of all sites showed no significant evidence of lysogeny (that is, FCIC values with bootstrapped 95% CIs that included zero: Famosa Slough, Lake Murray and Spanish Landing; Fig. 4b). When values ≤0 were excluded, site means increased and CIs constricted so as to give the appearance of significant levels of lysogeny at all sites (Fig. 4b). Similar to the published FCIC data sets, most FCIC values from our survey fell between 0 and 5% in a normal distribution centred around zero (Fig. 4c), indicating the true distribution and extent of the excluded data in Fig. 3b.

Experimental manipulation and FCIC. To probe the potential effects of host density and host growth rate on FCIC, we estimated FCIC in communities in technical replicates diluted either with nutrient-free buffers (low growth) or filtered site water (high growth). It was expected that higher dilutions would lead to

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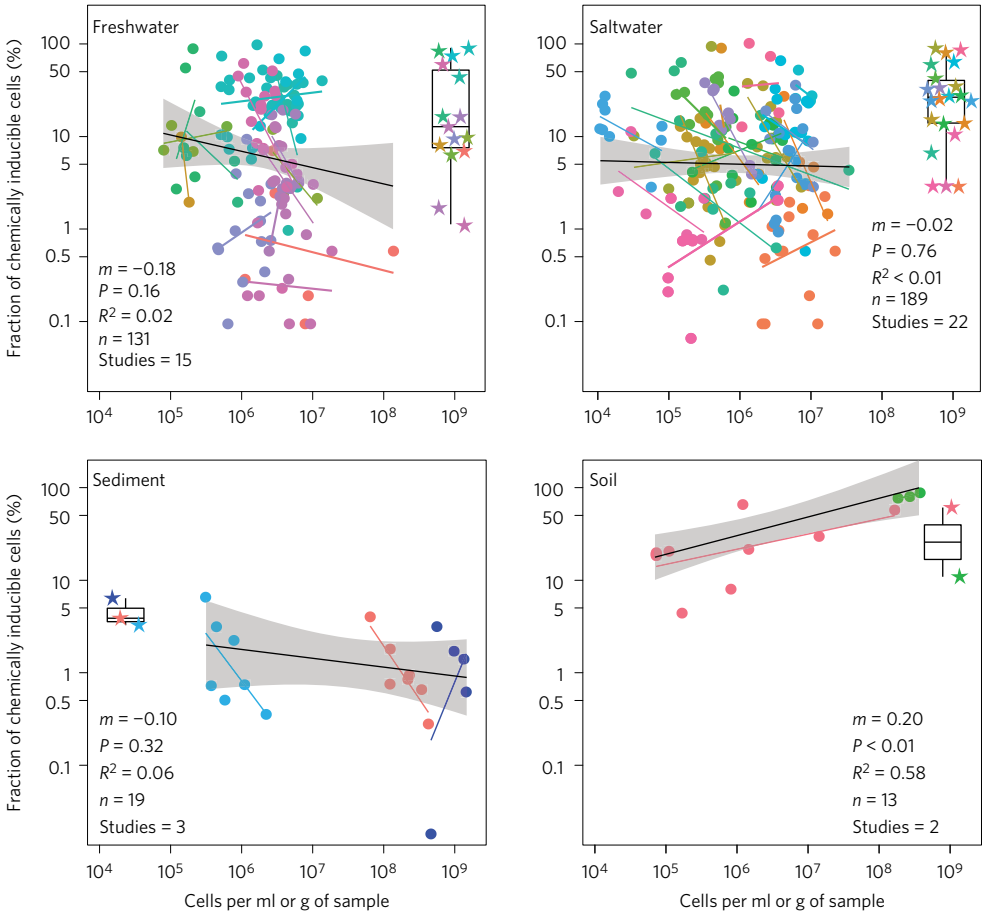

Figure 2 | Reported fractions of chemically inducible cells (FCIC, %) in freshwater, saltwater and sediment environments, showing a lack of negative host density dependence, and soils, showing positive host density dependence (cells per ml or g of sample). Host density (cells per ml or g of sample) and FCIC are both log-transformed. Black lines: across-study lines of best fit (black). Grey shading: 95% prediction intervals. Summary statistics (*m*, *P*, R^2 and *n*) are from linear regression across all data within each environment. Data points and associated linear regression lines of best fit from individual studies are colour-coded and do not show prediction intervals (see Supplementary Table 1 for details of each study). Boxplots show the distribution of ranges from individual studies within each environment (stars colour-coded by study). Boxplots are shown in sediment and soil environments for consistency, despite the low sample numbers. Boxes show the 25th, 50th (median) and 75th percentiles and whiskers range from minimum to maximum values. Data points from each reference are plotted individually in Supplementary Fig. 1.

artificially increased FCIC estimates due to a greater sensitivity to mitomycin C (ref. 16) and increased growth rates[27], especially in the higher-nutrient-site water dilutions compared to buffer dilutions.

Neither dilution series yielded monotonic changes in FCIC; instead, there was a dramatic and inconsistent increase in FCIC variability (Fig. 5a,b) compared to the undiluted technical replicates (Fig. 5c). Buffer dilutions of samples from Chollas Reservoir, Famosa Slough, Lake Murray, Old Mission Dam, Spanish Landing and Vacation Road sites showed ranges of 39.19, 16.17, 22.70, 19.58, 38.39 and 15.39% FCIC, respectively, and the corresponding site water dilutions were 32.73, 24.03, 13.19, 16.88, 17.86 and 56.36% FCIC (Fig. 5c). Variability was not consistent with either dilution or diluent at most sites (Fig. 5a,b). Furthermore, variation (range) within the diluted technical replicates from four of six sites (buffer dilutions: Chollas Reservoir and Spanish Landing; site water dilutions: Chollas Reservoir and Vacation Road) exceeded that observed across sites in half of the published freshwater and saltwater studies (Fig. 5c), suggesting that variability in local dynamics equals the impact of broader ecological drivers on FCIC. Compared to the 'correct' undiluted sample, dilution can drive up to 39.19% overestimates or 38.39% underestimates of lysogeny, with bias measured as deviation from the undiluted samples (Fig. 5a,b; median change from undiluted samples ± 12.85% FCIC with dilution). As above, FCIC values showed a normal distribution during dilutions (Fig. 5d). A third of the FCIC values measured with (Fig. 5a,b) and without (Fig. 4a) experimental manipulation were ≤0 (25 of 74 values, 33.78%) and thus would normally have been excluded (Fig. 3b).

Discussion

Host density as a driver of lysogeny in published data sets. The relationship between FCIC and host density at the global (Fig. 1b), within-environment (Fig. 2) and within-study (Fig. 3a) levels was almost universally highly variable and non-significant. Only a few studies show increasing FCIC at low host densities[16,17,24–26]. In soils, the relationship between FCIC and host density was constrained ($R^2 = 0.58$), significant ($P < 0.01$) and positive ($m = 0.20$), contrary to the current paradigm. Because analysis of aggregated data sets is predisposed towards finding significant relationships[28] and because we conducted regressions without alpha values corrected for our legion analyses (for example, ~50 linear regressions), the lack of significance here is robust evidence against a global, environmental or within-study level dependence of FCIC on host density. Increased sampling was associated with increased variability at both the within-study and across-study levels, even though the vast majority of studies

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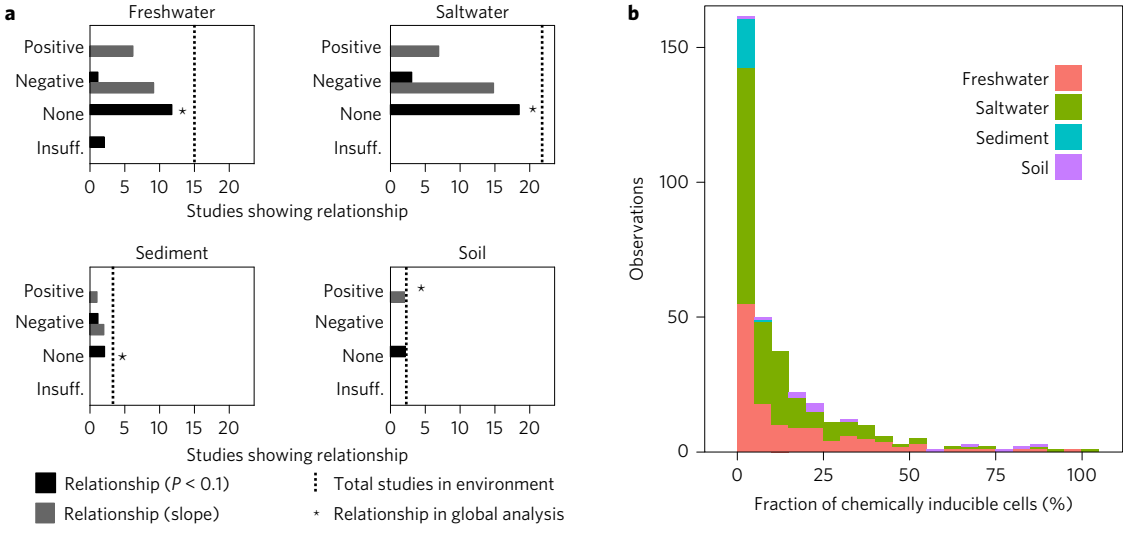

Figure 3 | Relationships between FCIC and host density at the within-study level and the truncated distribution of published FCIC values. a, Summary of the number of positive, negative and flat relationships observed in individual studies within freshwater ($n = 15$), saltwater ($n = 22$), sediment ($n = 3$) and soil ($n = 2$) environments. Black bars: significant relationships. Insuff: insufficient data, studies with $n < 3$. Grey bars: slopes. Stars: 95% confidence level drawn from the environment-level linear regressions shown in Fig. 2. Dashed vertical lines: total number of studies in that environment. **b**, Stacked histogram of FCIC values (in 5% bins) with the number of observations for each bin, colour-coded by environment ($n = 352$). Data points from each reference are plotted individually in Supplementary Fig. 1.

focused on two geographic areas (Supplementary Table 1 and Fig. 1). Ultimately, multiple decades to centuries of further research would be required to obtain a reliable host density dependence in FCIC (positive or negative), based on current rates of data accumulation in the twenty-year history of the field (as shown by power analysis). This lack of support for the current density-dependence paradigm invites the search for unidentified drivers of environmental lysogeny.

The 'silent third' of FCIC values ≤0. FCIC values ≤0 made up one-third of the FCIC values (the 'silent third') we observed and were (1) remarkably inconsistently distributed, with some technical replicates showing inhibition while others did not, and (2) ubiquitous across a range of host densities and sites (Figs 4a and 5a). Because FCIC cannot logically be less than zero, these frequent negative FCIC values at the community level show an abiding disassociation of lysogeny and FCIC. Combined with the capacity for excluding these values to skew estimates of lysogeny (Fig. 4b), the distribution of FCIC values ≤0 suggests that there may be pervasive bias in the characterization of lysogeny and its drivers so far.

Origins of the 'silent third' of FCIC values ≤0. Mitomycin C induction is a highly dose-dependent approach. FCIC values ≤0 arise when induction treatment yields unchanged or lowered viral densities ($V_i \leq V_c$ in equation (1), Table 1). While an unchanged viral density (FCIC = 0) may accurately reflect the absence of lysogens if mitomycin C is correctly dosed, it may also indicate unsuccessful induction (that is, an absence of induction whether lysogeny is present or not) if mitomycin C is underdosed, yielding a false negative (Type II error, Table 1). FCIC values <0 ($V_i < V_c$) are more consistent with mitomycin overdose causing inhibition than unsuccessful induction[6] (Table 1), although these 'silent third' of values are commonly treated similarly to FCIC = 0 values and excluded as unsuccessful induction[13]. Although mitomycin C dosage may be tailored in single-strain systems, when dosing mixed communities an unknown proportion of organisms or lineages may be underdosed, overdosed or correctly dosed at any given dose[11]. Although it is unclear what any FCIC value really means, the observed variability in FCIC may be indicative of the taxonomic or physiological state of organisms in a given sample[29,30].

Induction in mixed communities versus isolates. Although lysogeny appears to be a variable phenomenon, the majority of sequenced bacterial genomes contain prophages[5], and previous investigations reported 43% (ref. 10), 25–62.5% (ref. 4) and 71% (ref. 27) of bacterial isolates to be inducible with mitomycin C. Two-thirds of bacterial viruses with known lifestyles and sequenced genomes are temperate[31]. In comparison with these figures, the 0–5% mode of FCIC values (Fig. 3b) suggests that mitomycin C induction underestimates community-level lysogeny relative to strain-level analyses, an effect probably masked by exclusion of the 'silent third' (Fig. 4b). Inclusion of the 'silent third', combined with the use of more diverse inducers[10,32,33] and further investigation of the linkages between lysogeny and mitomycin C induction, as well as host density[13], identity[30] and growth rate[27], may reconcile FCIC and lysogeny.

Stochastic effects of dilution on FCIC. We induced lysogens in diluted samples to probe the potential systematic impacts of host density on FCIC estimation in slow- (buffer diluent) and fast- (site water diluent) growing communities. Mitomycin C was added simultaneously with diluent, precluding changes in lysogeny during induction. Regardless of diluent, the most marked effect of dilution was a large and inconsistent increase in the variability in FCIC between replicates compared to undiluted replicates or published studies (Fig. 5a,b versus Fig. 4a). Dilution can result in lowered taxonomic diversity, but with variable changes in functional capability[34–37], making stochastic functional changes in microbial communities with dilution a candidate for further investigation.

Dilution stochasticity and viral production assays. When dilution is used to derive estimates of lytic and lysogenic viral production, rates of lysogeny are assumed to remain unchanged (for example, ref. 38). However, if the variation in FCIC with dilution observed here (Fig. 5) is real, changing rates of lysogeny during dilution will methodologically bias dilution-based estimates of viral

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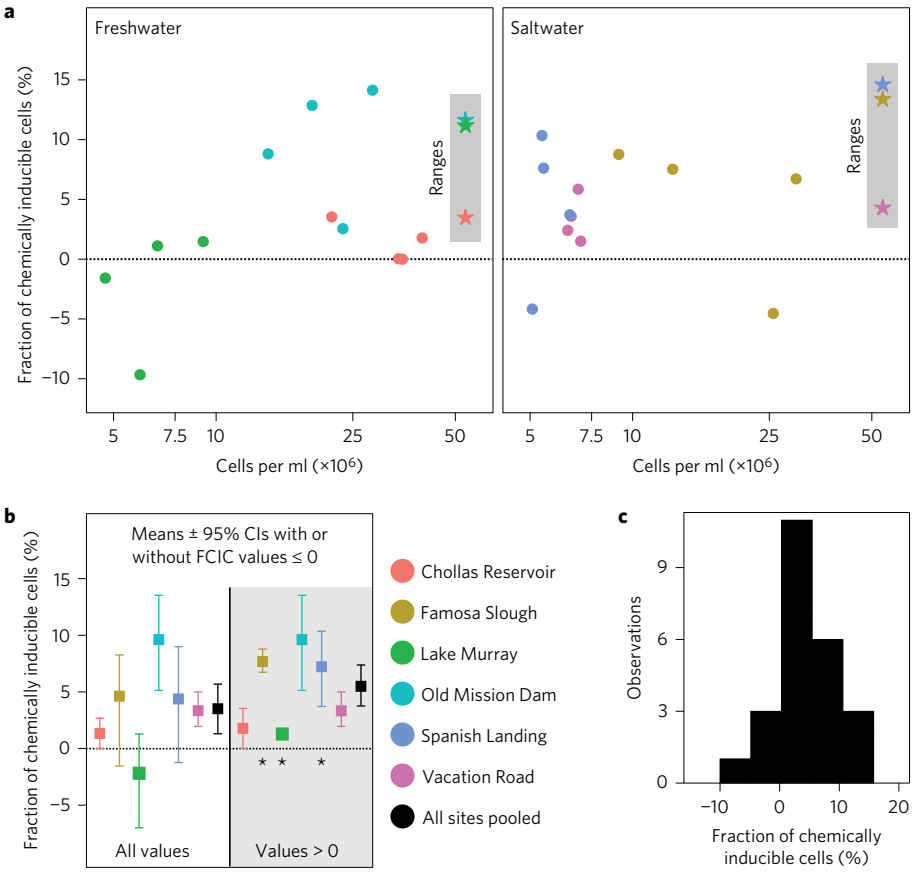

Figure 4 | FCIC variability between technical replicates, within sites and between sites, with significant impacts of excluding values ≤0 in most sites. **a**, FCIC from technical replicates ($n = 4$) across three freshwater and three saltwater sites (colour-coded by site; host density log-transformed). Grey shaded areas: stars show ranges of FCIC values in each environment. **b**, Comparison of FCIC means and confidence levels with (unshaded area; $n = 4$ for all sites) or without (shaded area; $n = 2$ in Lake Murray samples; $n = 3$ in Chollas Reservoir, Spanish Landing and Famosa Slough sites; $n = 4$ in Old Mission Dam and Vacation Road sites) FCIC values ≤0. Squares: means. Whiskers: bootstrapped 95% CIs. Colour-coded by site, with global mean ± 95% CIs also shown (black). Asterisks: sites where exclusion of FCIC values ≤0 significantly alters evidence of the presence or absence of lysogeny ($P < 0.05$; 95% CIs do or do not cross zero). **c**, Histogram (5% bins) of the distribution of replicate FCIC values from these sites, showing an approximately normal distribution centred around the 0–5% FCIC bin ($n = 24$).

production. By contrast, if rates of lysogeny remain constant during dilution, then the observed variability reveals a divergence of actual and estimated lysogeny (FCIC). Pairing FCIC and dilution-based viral production estimates in future studies may characterize the conditions that drive disassociation of FCIC from lysogeny.

The 'lurking variable' that drives FCIC. Although FCIC was not driven by host density, the FCIC distribution was constrained and consistent across published studies (Fig. 3b), undiluted samples (Fig. 4c) and diluted samples (Fig. 5c). FCIC thus appears to be a non-random parameter possibly driven by environmental condition(s). Observations in this study suggest that the 'lurking variable' (or variables) (1) varies significantly between sites, unrelated to abiotic conditions such as temperature and salinity; (2) does not vary in soil but is variable in particulate sediment and aquatic environments; (3) varies inconsistently with dilution; (4) is conserved across a range of microbial densities; and (5) does not correlate with host density or growth rate. Many variables fit this description, including community taxonomic, metabolic and functional composition (for example, refs 29, 30), possibly varying by environment. While techniques such as metagenomics provide both community profiling and the identification of prophage elements[39,40], observational approaches alone cannot identify which variables determine the prevalence of lysogeny. Here, we have combined observational and experimental approaches to capture the variability of FCIC and profile its driver(s), while eliminating canonical determinants such as host density, to inform future studies using mitomycin C inductions.

Conclusions

We have examined the evidence of host density dependence in inductions-based studies to determine whether the paradigm that lysogeny is promoted at low host density is generally supported, or whether the recently proposed 'piggyback-the-winner' model of high-density lysogeny[1] is actually consistent with inductions data sets. Rather than supporting either model, the analysis showed high variability and a lack of density dependence (with the exception of soils, where 'piggyback-the-winner' was supported) in FCIC estimates.

No transition—as suggested by earlier FCIC studies and lytic modelling efforts—from low-density lysogenic to high-density lytic dominance is supported. The observed issues hinder the use of FCIC in describing the lytic–lysogenic switch and may have impeded earlier attempts to understand patterns of environmental lysogeny (for example, ref. 43) while aiming to resolve the broadly observed sublinear power-law relationship between viral and host densities[1,41–43]. Despite high variability, FCIC values showed markedly constrained distributions when plotted against host density,

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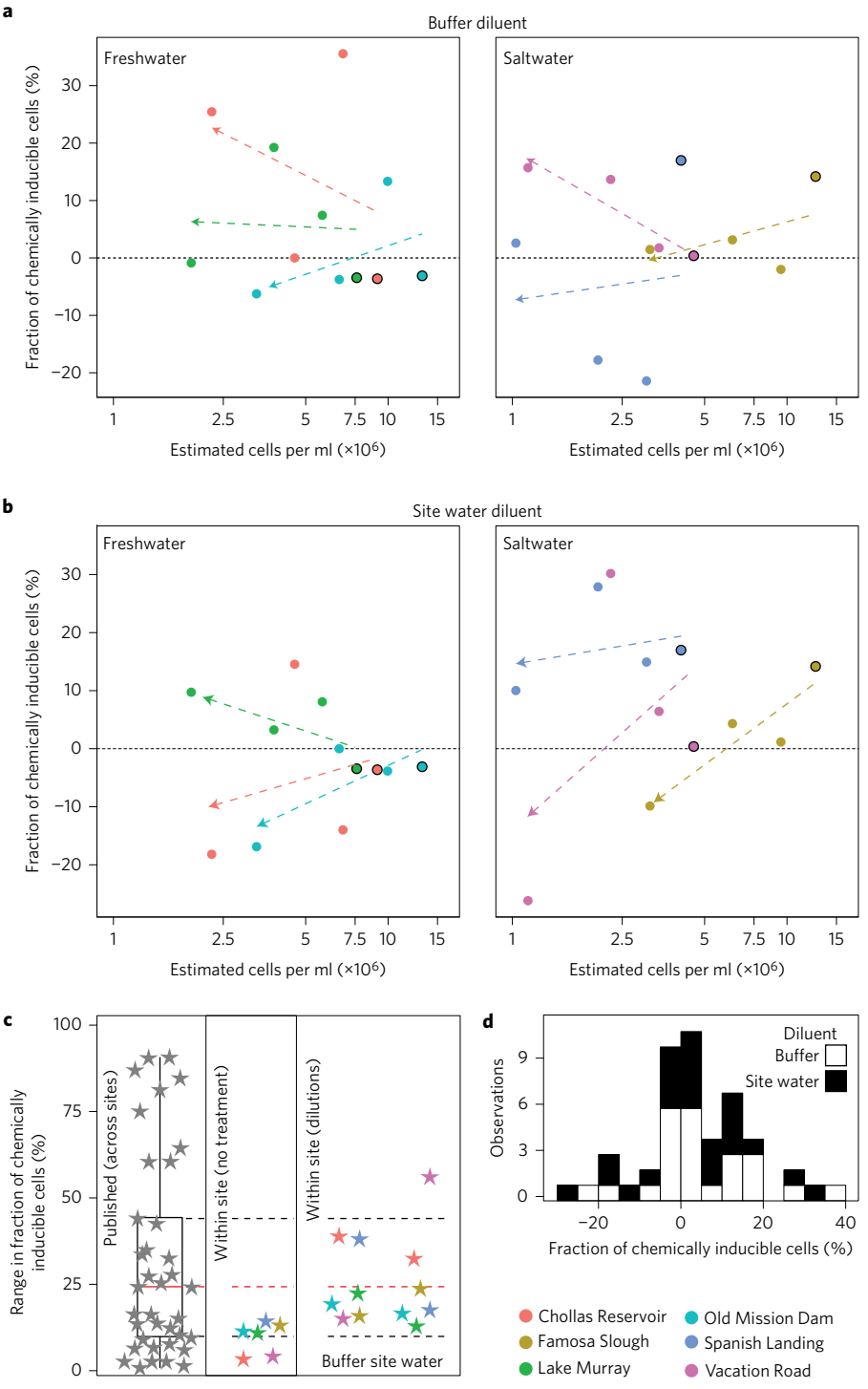

Figure 5 | Effect of dilution on variability in FCIC. a,**b**, FCIC plotted against cell density following dilution with 0.02-µm-filtered buffer (**a**) or 0.02-µm-filtered site water (**b**). Dashed heuristic arrows: lines of best fit across technical replicates, colour-coded by site, pointing towards more diluted samples. Black rings: 'correct' undiluted samples, $n = 1$ for each data point shown. **c**, FCIC ranges from published studies and both undiluted (no treatment) and diluted samples from this study ($n = 4$ for each range from this study; sample numbers for each published range are provided in Supplementary Table 1). Boxplot: FCIC ranges from individual published studies (grey stars) from freshwater and saltwater environments. Dashed lines: extensions of published median value and 25th and 75th percentiles across the undiluted and diluted samples analysed here. The majority (66%) of undiluted technical replicates show ranges in the 25th to 50th percentiles of published studies using biological replicates and addressing FCIC across sites. **d**, Histogram (5% bins) of the distribution of replicate FCIC values from the dilution experiments, showing an approximately normal distribution centred around zero (buffers, white; site water, black).

indicative of a Simpson paradox in which unexamined variables are driving FCIC (ref. 44). Probing of this variability revealed profound artefacts resulting from exclusion of FCIC values ≤0 in published studies and also allowed profiling, but not identification, of the possible driver. Although it is currently unclear what FCIC values mean, the variability in FCIC reported here may guide future studies elucidating what now appears to be a nascent driver of viral dynamics.

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Methods

Mining published values. Published values of the fraction of chemically inducible cells (FCIC, %) were taken from 39 studies mined from Google Scholar that reported host densities and corresponding FCIC values estimated using induction by the addition of mitomycin C (Supplementary Table 2, Supplementary Fig. 1 and Supplementary Source Data)[11,12,14–17,24–26,29,30,32,45–70]. Google Scholar hits to the search 'mitomycin C induction lysogeny' were mined to the tenth page of the search engine results. Supplementary probing of papers citing Jiang and Paul[10] showed a high degree of redundancy to the Google Scholar approach and was considered evidence that the majority of papers satisfying the SYBR and mitomycin C methodological criteria had been retrieved. Where necessary, values were extracted from figures using the webplotdigitizer Chrome extension tool (http://arohatgi.info/WebPlotDigitizer/app/). Most papers did not report FCIC if induced viral densities did not exceed untreated viral densities, as this would yield FCIC estimates ≤0, which are interpreted as unsuccessful induction[13]. Published FCIC data sets were therefore analysed with values ≤0 excluded to ensure consistent analysis. All within-study and global across-study linear regressions were conducted using data at the greatest resolution possible. Individual measures were used whenever accessible, and mean values otherwise (Supplementary Table 1). Where FCIC estimates were provided using minimum and maximum burst sizes (for example, refs 12,30), minimum values were used.

FCIC estimation. Mitomycin C from a stock less than 1 month old, suspended in Sigma water (Sigma-Aldrich) was added to environmental samples to a final concentration of 1 μg ml^{-1}, consistent with most environmental induction studies (Supplementary Source Data) and as prescribed for high-density near-shore samples[13]. Sigma water was substituted for control samples to ensure a similar dilution to induced samples. Samples, with corresponding mitomycin-C-negative controls, were then incubated for 18–24 h at room temperature in the dark, and viral densities were then compared between mitomycin-C-positive treatments and mitomycin-C-negative controls. FCIC values were then calculated from equation (1)[11–13] using a burst size of 25 to approximate the median value of burst sizes used in published studies that did (median burst size 22.5) and did not (median burst size 30) vary burst sizes for each sample, respectively (Supplementary Source Data).

***In situ* studies.** The following freshwater and saltwater sites around San Diego, California, USA (Supplementary Table 2) were sampled with sterile 50 ml polypropylene tubes: Famosa Slough, Spanish Landing, Vacation Road, Chollas Reservoir, Old Mission Dam and Lake Murray. Samples were stored in the dark at room temperature and processed within 2 h of collection. Subsamples were aliquoted into 2 ml technical replicates in 24-well plates (technical replicates are ecological pseudoreplicates as they are intentionally not independent samples; Corning). The three technical replicate fractions required to estimate FCIC (initial cell counts, final mitomycin-C-positive viral counts and final mitomycin-C-negative control viral counts) for each technical replicate were transferred within one syringe-draw to keep technical replicate fractions as coupled as possible. Initial counts were then conducted, and mitomycin-C-positive and mitomycin-C-negative samples treated. Mitomycin-C-positive and mitomycin-C-negative samples were segregated in different plates to preclude airborne antibiotic impacts on controls. After incubation for 18–20 h (ref. 13), 1 ml samples were drawn from each 2 ml well, fixed with paraformaldehyde (2% final concentration) for 30 min, and then flash-frozen in liquid nitrogen[1,71]. Samples were thawed at room temperature immediately before staining with 2× SYBR Gold nucleic acid stain (Life Technologies) for 30 min and filtered onto 0.02 μm Anodisc filters (Whatman)[1,71]. Filters were mounted on slides and imaged on an Olympus ×100 object magnification oil-immersion microscope, and counts were conducted using Image Pro software (Media Cybernetics), with observers blind to the sample identity until statistical analysis.

Dilution experiments. Sites sampled in the observational study were resampled for manipulative dilution experiments. All sampling and aliquoting procedures were the same as above. However, rather than aliquoting 2 ml of subsampled site water into each well of the 24-well plates, technical replicates were diluted to produce undiluted, 25, 50 and 75% dilutions of unfiltered water samples (undiluted, 75, 50 and 25% unfiltered site water, respectively). This was done by adding either 0.02-μm-filtered buffer (buffer dilutions, Fig. 5a; artificial seawater buffer (Tropic Marin, Germany) at saltwater sites or Hydra Media buffer[72] at freshwater sites) or 0.02-μm-filtered site water (site water dilutions, Fig. 5b). Host densities in each dilution were estimated as host density in undiluted samples when mitomycin C was added multiplied by the dilution factor. Both artificial seawater and freshwater diluents maintain chemical buffering of site water, but do not contain nutrients to sustain or enhance host growth. As a result of this, host density was diluted, with probable concomitant simplification of community composition and elevation of the mass action dose of inducing agent per cell present, while the rates of lysogeny and host growth rate were presumably unchanged. Dilution with site water dilutes host densities but does not change nutrient availability, allowing host growth to increase in proportion to dilution. Undiluted samples (0% dilutions) were considered 'correct' estimates of FCIC for comparison, because they are equivalent to samples typically processed for published FCIC studies. Mitomycin C addition, incubation, sample fixation, storage and processing were the same as above, but slides were imaged using an Olympus ×60 objective magnification oil-immersion microscope.

Statistical analysis. All statistical analyses were conducted with a conventional *a priori* alpha of 0.05 (95% confidence). Despite the large number of analyses conducted herein (for example, ~50 linear regressions), no correction was applied to the alpha to facilitate identifying significant relationships between FCIC and host density, even at risk of incurring Type I error. Linear regressions were conducted using the lm() function in R (reported in the plots in Figs 1–3). These were complemented by ordinary least squares (OLS) Type II linear regressions using the lmodel2() function of the lmodel2 R package across all data and across freshwater, saltwater, sediment and soil environments, to ensure variability in host density measurements was not skewing our analyses. Published data sets showed a negative relationship between n and R^2, suggesting that the analysis of means, as the majority of data available for this study, facilitated finding relationships between FCIC and host density (Supplementary Table 1). Power analysis using a test for correlation were conducted using published data sets (Supplementary Source Data) with the pwr.r.test() function in R to estimate the sample sizes needed to obtain a power of 0.8 with a significance level of 0.05 in a two-tailed test. As the FCIC values generated here were intentionally not independent (that is, they were technical, not biological, replicates), a conservative non-parametric bootstrapping approach was used to generate means and 95% CIs (Fig. 4). These parameters were estimated using the boot_out() function in R with 10,000 iterations on data with all FCIC values included as well as with FCIC values ≤0 excluded, consistent with the literature.

Data availability. Published data subjected to meta-analysis are provided in the Supplementary Source Data. These data and experimental data sets are also available from the corresponding author upon request.

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Author contributions

B.K. and F.R. designed, conducted and wrote up the study. B.B., L.B., M.B., A.C.-G., J.d.C., R.E., B.F., J.G., A.H., P.K., L.W.K., A.L., J.N., G.P., L.P., N.R., S.S., A.S., C.S. and M.Y. contributed data, analysis and manuscript preparation.

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Correspondence and requests for materials should be addressed to B.K. and F.R.

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Competing interests

The authors declare no competing financial interests.

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