Understanding policing as a mechanism of cheater control in cooperating bacteria

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Keywords: individual-based simulation, public goods dilemma, evolution of cooperation, sociomicrobiolgy

Short title: Cheater control in cooperating bacteria

Abstract

Policing occurs in complex insect, animal and human societies, where it is used as a conditional strategy to prevent cheating and enforce cooperation. Recently, it has been suggested that policing might also be relevant in enforcing cooperation in much simpler organisms such as bacteria. Here, we used individual-based modelling to develop an evolutionary concept for policing in bacteria, and identify the conditions under which it can be adaptive. We modelled interactions between cooperators, producing a beneficial public good, cheaters exploiting the public good without contributing to it, and public-good-producing policers that secrete a toxin to selectively target cheaters. We found that toxin-mediated policing is favored when (i) toxins are potent, (ii) cheap to produce, (iii) cell and public good diffusion is intermediate, and (iv) toxins diffuse farther than the public good. Overall, we show that toxin-mediated policing can enforce cooperation, but the parameter space where it does so is quite narrow. Moreover, we found that policing decays when the genetic linkage between public good and toxin production breaks. This is because policing is itself a public good, offering protection to toxin-resistant mutants that still produce public goods, yet no longer invest in toxins. Our work suggests that very specific environmental conditions are required for genetically fixed policing mechanisms to evolve in bacteria, and offers empirically testable predictions for their evolutionary stability.

Introduction

Cooperation, a process where individuals act to increase the fitness of others at an immediate cost to themselves, is common across the tree of life [1, 2]. While cooperation is widespread in humans [3] and higher organisms such as insects and vertebrates [4, 5], we have only recently recognized that also microbes have evolved adaptive cooperative behavior [6-9]. Types of microbial cooperation include the formation of biofilms, where individuals secrete polymeric compounds to build a protective extracellular matrix [7], the formation of fruiting bodies where some individuals sacrifice themselves to enable the dispersal of others [10]; and the secretion of shareable metabolites, such as proteases to digest nutrients [11, 12], siderophores to scavenge extra-cellular iron [13, 14], and biosurfactants enabling group motility [15].

Although cooperation is thought to provide benefits for the collective as a whole, it is intrinsically vulnerable to exploitation by cheating mutants, which benefit from the cooperative acts performed by others, but refrain from contributing themselves to the welfare of the group [16]. Thus, there has been great interest in identifying mechanisms that maintain cooperation and prevent the invasion of cheaters [2, 17, 18]. Work on microbes have proved particularly useful in this context because cheating mutants can easily be engineered and factors important for cooperation can be experimentally manipulated in laboratory settings. Studies following this approach revealed a plethora of mechanisms promoting cooperation, including: (i) limited dispersal ensuring that cooperators stay together [19, 20]; (ii) molecular mechanisms allowing the recognition of other cooperators [21-23]; (iii) regulatory linkage between multiple traits imposing additional costs on cheaters [24-28]; and (iv) mechanisms

reducing the cost of cooperation such as public good recycling [29] or the use of superfluous nutrients for public goods production [30, 31].

In addition, several studies suggested that bacteria have evolved policing mechanisms, which enable cooperators to directly sanction cheaters and thereby enforce cooperation [32-36]. For example, Wang et al. [34] showed that in the opportunistic pathogen *Pseudomonas aeruginosa*, the synthesis of a publically shareable protease is regulatorily coupled to the synthesis of the toxin cyanide in such a way that cheaters, deficient for protease production, simultaneously lose immunity against cyanide. Thus, the costly synthesis of a harmful toxin, which selectively targets non-cooperative cheaters, can be understood as a policing mechanism. While it seems intriguing that organisms as simple as bacteria can perform policing behavior, multiple questions regarding the evolution of microbial policing have remained unaddressed. For one thing, we know little about the environmental conditions promoting policing via toxin production. In this context, one would expect that the spatial structure of the environment, which affects the diffusion of cells, public goods and toxins, should play a crucial role [37-39]. Moreover, it is unknown how potent a toxin must be and how much it can cost in order to efficiently fight cheaters. Finally, it remains unclear whether policing is an evolutionary stable strategy or whether non-policing cooperators, which are immune against toxins can exploit policers as second order cheaters [33], as it is the case in higher organisms [40].

Here, we address these issues by using realistic individual-based models that simulate interactions between cooperating, policing and cheating bacteria on a toroidal two-dimensional surface [39]. In our *in-silico* approach, bacteria are modeled as discs and seeded

in low numbers onto the surface of their habitat, where they can consume resources, grow, divide, disperse, and secrete compounds according to specified parameters. Important to note is that both bacteria and public good molecules are modelled as individual agents and are free to diffuse on a continuous landscape, closely mimicking natural conditions. We considered four different bacterial strain: (1) a wildtype cooperator strain producing a beneficial public good; (2) a policing strain producing a toxin and an immunity protein in addition to the public good; (3) a cheating strain neither producing the public good, the toxin nor the immunity protein; and (4) a cooperator strain producing the public good and the immunity protein, but not the toxin. We assume tight linkage between all three traits, such that (3) directly evolves from (2), matching the policing mechanism proposed by Wang et al. [34].

In a first set of simulations, we examined the performance of the cooperator, cheater and policing strains in monoculture to understand how the relative costs and benefits of public good and toxin production affect strain fitness. Next, we competed wildtype cooperators against cheaters across a range of bacterial and public good diffusion coefficients to determine the parameter space where cooperation is favored in the absence of policing [39]. Subsequently, we competed the cheater against the policing strain across the same parameter space, to test whether policing extends the range of conditions across which cooperation is favored. Finally, we simulated the situation where toxin-resistant public good producers evolve from the policing strain, and ask whether policing via toxin production itself constitutes an exploitable public good.

Results

The boundary costs of policing vary in response to environmental diffusivity

To assess how costly policing can be in order to still generate a net benefit of cooperation, we compared the growth of the policing strain (*P*) in monoculture, to the growth of the wildtype non-policing cooperator (*W*) and the strain producing neither public goods nor policing toxins (*C*). As expected, *C* was not affected by the diffusivity of the environment, but grew at a constant rate determined by its basic growth rate μ, reaching carrying capacity after 12,000 time steps (Fig 2). Conversely, the growth of *W* was reduced in more diffusive environments, where the likelihood of public good sharing and consumption declines. Nonetheless, *W* grew significantly better than *C* under all conditions, demonstrating the benefit of public goods cooperation (Fig 2). The performance of *P* greatly varied both in response to the relative costs of policing (c_{pg}/c_{tox}) and the diffusivity of the environment. In environments characterized by low diffusion, *P* always outperformed *C* even when the cost of toxin production was four times higher than the cost of public good production (Fig 2A). This was no longer the case in environments characterized by intermediate or high diffusivity, where *P* only grew better than *C* when toxins were cheaper to produce than public goods (Fig 2B and 2C). If this condition was not met, then the high costs of policing combined with reduced public good consumption decreased cell growth to a point that prevented populations from reaching carrying capacity. Based on these results, we chose c_{pg}/c_{tox} = 1.5 for all subsequent simulations.

Interestingly, the relationship between the cost ratio $r = c_{pg}/c_{tox}$ and the time to reach carrying capacity (τ_K) was best captured by the Monod equation [41], a hyperbolic equation initially used to explain the exponential growth rate as a function of nutrient concentration.

Applied to our system, we found that the function

$$R(\tau_K) = R_K \frac{\tau_K}{R_\tau + \tau_K}, \quad (1)$$

provides a fair approximation to relate r to τ_K, where R_K represents the ratio limit for reaching carrying capacity and R_τ the time at which the carrying capacity is half the maximum (Fig S1).

However, the equation does not fully capture the observed pattern when the ratio approaches one, namely when $c_{tox} \approx c_{pg}$, under conditions of low environmental diffusivity. This is partially understandable since $R(\tau_K)$ does not directly take the viscosity of the medium into account.

In the absence of policing, cooperation is favored when cell diffusion is low

When competing *W* against *C* without a policing mechanism in place, cooperation was only strongly favored when cells did not diffuse (Fig 3A). In other words, if cooperator cells grew as microcolonies, physically separated from the cheaters, then efficient public good sharing among cooperators is promoted (see [19, 42, 43] for experimental support). We further found that cooperators and cheaters could coexist when cell and public good diffusion was low, but greater than zero (Fig 3A). Under all other conditions, cheaters strongly dominated and pushed cooperators to very low frequencies. These results are in line with our previous simulations, showing that there is only a narrow parameter space within which public good cooperation can be favored [39].

Policing through a potent toxin extends the parameter space favoring cooperation

The introduction of a policing mechanism, which operates via the secretion of a toxin that selectively targets cheaters, had multiple dramatic effects on the competitive outcome

between the cheater *C* and the cooperating policer *P* (Fig 3B). With a potent toxin (θ_T = 1000), policing strongly increased selection for cooperation under conditions where cooperators could previously only coexist with cheaters (compare Fig 3A and 3B for combinations of low public good and cell diffusion). This demonstrates that policing can indeed extend the parameter space across which cooperation can be favored. Conversely, we found that policing also had negative effects and drastically accelerated selection against cooperation (Fig 3B), especially under conditions where cheaters previously experienced only a moderate selective advantage (compare Fig 3A and 3B for combinations of intermediate public good and cell diffusion). These two opposing effects led to a sharp transition between conditions that either completely favor *P* or *C*, leaving very few conditions where coexistence between the two strains is possible.

Toxin diffusion and potency affect selection for cooperation

We found that toxin diffusion significantly affected the parameter space across which cooperation was favored (Fig 3B and 3C). While we manipulated toxin diffusion across a gradient from 1 to 7 $\mu m^2/s$ in steps of 1, we here only present the two extremes (Fig3B with d_t = 1, and Fig 3C with d_t = 7), as the outcomes of the other treatments resulted in intermediate patterns. Our simulations revealed that increased toxin diffusion was favorable for cooperation, especially under conditions of relatively high public good diffusion but low cell diffusion. These results show that policing is particularly efficient when toxins are sent away to target more distant competitors whilst keeping the public good more locally for sharing among clonemates.

Our simulations further revealed that high toxin potency is crucial for policing to promote cooperation (Fig 3D and 3E). While this finding seems trivial at first sight, the dramatic effects we observed when decreasing toxin potency are remarkable. In our simulations, we manipulated toxin potency by varying θ_T, the number of toxin molecules sensitive cells can tolerate before they die (θ_T varied from 1000 to 2200 in steps of 400). Important to note is that larger values of θ_T did not only increase toxin tolerance, but also the latency phase during which toxins had little effects on growth (see equation 3). Given this context, we found that a reduction of toxin potency by 45 % (Fig 3D, θ_T = 1000 versus Fig 3E, θ_T = 1800) already negated any benefit of policing, and even increased the parameter space across which cooperation was selected against.

Policing is not an evolutionary stable strategy if genetic linkage between traits breaks

In a next set of simulations, we asked whether policing is an evolutionary stable strategy or whether it can be invaded by a non-policing strain that produces the public good, but is resistant against the policing toxin (*R*). Such mutant strains can arise when the genetic linkage between the three traits (public good, toxin, immunity) is broken. We simulated this situation by competing the policing strain *P* against *R* in the presence of the cheater *C* in nine environments differing in their diffusivity (Fig 4). Here, we focused on a subset of combinations of diffusion coefficients (D_c= 1, 1.5, 2; d_p= 3, 4, 5; and d_t= 7 μm^2/s). We chose this parameter space because it comprises all possible outcomes from the competitions between *P* and *C* (Fig 3C: five cases of *P* dominance, two cases of *C* dominance, and two cases of coexistence).

We found that the addition of *R* to the system consistently drove *P* to extinction regardless of the diffusivity of the environment (Fig 4). Our simulations, which kept populations at half the carrying capacity (*K*/2 ~500 cells) for an extended period of time (roughly 10 times longer than in the previous pairwise competition assays), allowed us to distinguish three distinct competition phases. The first phase comprises the time frame in which the community grows from three cells to *K*/2. During this phase, we observed cyclical fluctuations of strain frequencies with a general tendency for *C* to increase, *P* to decrease, and *R* to remain stable. The cyclical patterns observed here are reminiscent of the rock-paper-scissors dynamics described by previous studies on bacterial interactions [44-46], where strains chasing each other in non-transitive interaction patterns with no overall winner. The second phase was characterized by a pronounced dip in *C* frequency accompanied by a strong increase in *R* frequency, and a moderate increase in *P* frequency in eight of the nine diffusion conditions (Fig 4). This pattern is most likely explained by the accumulation of toxins in the environment, which efficiently suppressed *C*, but at the same time gave *R* leverage, as it could benefit from the effect of policing without paying the cost for it. During the third phase, we observed the extinction of *P*, the concomitant recovery of *C* followed by a decrease of *R* (Fig 4). These patterns arise because as *P* decreases, toxin concentration declines allowing the recovery of *C*, which then efficiently exploit *R*. In all cases, our simulations returned to a simple cooperator-cheater scenario, with the relative success of the two strategies being determined by the diffusivity of the environment (as shown in Fig 3A).

Discussion

Understanding how policing can repress competition and foster cooperation in social groups of higher organisms has attracted the attention of evolutionary biologists for decades [2, 47-

53]. Here, we developed a conceptual evolutionary framework to examine whether policing could also be an effective way to enforce cooperation in groups of microbes, sharing beneficial public goods. In our models, policing is exerted via the secretion of a toxin that specifically targets cheater cells, which do not contribute to the pool of public goods. Our simulations reveal that policing is most conducive under conditions of intermediate cell dispersal and public good diffusion, where it can extend the parameter space, under which cooperation is favored. We further found that an effective policing mechanism entails a toxin that is: (i) cheaper to produce than the public good; (ii) very potent in killing; and (iii) more diffusible than the public good in order to effectively reach cheaters. However, our simulations also reveal two major downsides of policing. First, policing can accelerate the loss of cooperation under conditions where cheaters and cooperators can coexist in the absence of policing. This leads to a sharp state transition between conditions where policing either favors or disfavors cooperation. Second, policing is not an evolutionary stable strategy if the genetic linkage between the production of the public good, and the toxin-anti-toxin complex can be broken. If this occurs then toxin-resistant mutants that produce public goods, but no longer contribute to toxin production, derail policing. This demonstrates that microbial policing itself constitutes an exploitable public good [33].

Microbiologists have recently proposed a number of potential toxin-based policing mechanisms that seem to keep cheaters in check [33-36]. While the idea of bacteria being able to punish cheating community members is exciting, our simulations reveal that the ecological spectrum under which policing can be favored is actually quite narrow. For one thing, we found that policing is not required when environmental diffusivity is low, conditions that promote cooperation *per se*. The reason for this is that low public good and bacteria diffusion

lead to significant spatial structuring of the bacterial community, separating cooperators from cheaters, which promotes the local sharing of public goods among cooperators [19, 20, 43, 54]. Moreover, we show that policing is not favorable when environmental diffusivity is high, conditions that break any spatial association between cooperators and their public goods. Toxin-mediated policing is detrimental here because: (i) cheaters can freely exploit public goods; (ii) many toxins get lost due to high diffusion, and thus never reach their target; (iii) and the high level of cell mixing reduces the efficiency and selectivity of policing, as cooperators are hit by toxins as often as cheaters [38]. Overall, it turns out that only intermediate levels of environmental diffusivity proved beneficial for policing. The issue with this narrow parameter space is that environmental diffusivity is likely to vary both temporally and spatially under natural conditions, which could quickly shift the selective balance for or against policing. It thus remains to be seen whether policing can indeed evolve under fluctuating environmental conditions.

Another important point that has received little attention so far concerns the question whether the reported policing mechanisms have indeed evolved for this very purpose or whether they represent by-products of regulatory linkage of traits for other reasons than policing [55]. For instance, in the case of *P. aeruginosa* it is well conceivable that cyanide primarily serves as a broad-spectrum toxin to target inter-specific competitors under high cell density [56]. This might be the reason why the expression of cyanide is controlled by quorum sensing, and why it is regulatorily linked to other public good traits, such as protease production, whose expression is also controlled in a density-dependent manner [57]. Consequently, the observed cyanide-mediated policing exerted by wildtype strains on protease-deficient strains could be a mere by-product of this regulatory linkage. Alternatively, it is also possible that an initial co-

incidental regulatory linkage between cyanide and protease later proved useful as a policing mechanism and evolved as such through cooption [58]. Clearly, further research is needed to uncover the evolutionary history of these putative policing mechanisms, and care must be taken to distinguish between mechanistic (proximate) and evolutionary (ultimate) explanation of observed behavioral patterns [55].

The potential policing mechanisms reported for microbes and the one implemented in our simulations differ in one important aspect from the policing systems found in higher organisms. Specifically, the difference is that the microbial policing mechanisms are genetically fixed (i.e. strains are either cooperating policers or cheaters), whereas in higher organisms policing is perceived as a conditional strategy, which can be applied to sanction cheaters only if required [2, 47-52, 59]. In the latter scenario, individuals can take decisions on whether to cheat or to cooperate, and whether to impose sanctions or not. In certain cases, it was found that the mere threat of policing was sufficient to coerce individuals to cooperate and prevent cheating in the first place [51, 53, 60]. Microbes clearly lack cognitive abilities for such conditional behaviors, and it is thus not surprising that cheating, cooperating and policing strategies are genetically fixed in these organisms. Nonetheless, we argue that it seems fair to use the term ‘policing’ for the reported behaviors, but also to keep in mind the difference between conditional and fixed strategies.

In summary, our work contributes to the development of an evolutionary concept for policing in microbial systems. It shows how ecological factors, in particular the diffusivity of the environment, interact with the properties of a toxin-mediated policing system, to define the parameter space in which policing can be favored. It further demonstrates how realistic

individual-based modelling, tracking both cells and their public goods over time and across space, can be used to deepen our understanding of social interactions in microbes.

Materials and methods

The in-silico *landscape and its digital bacterial inhabitants*

The *in-silico* habitat consists of a two-dimensional continuous toroidal surface, with no boundaries. The size of the surface is 60x60 μm^2 = 3,600 μm^2. Bacteria are modeled as discs with an initial radius of 0.5 µm. Bacteria can consume resources, which are unlimited in our system, grow at a basic growth rate, and divide when reaching the threshold radius of 1 µm. Bacteria can disperse on the landscape according to a specific cell diffusion coefficient D ($\mu m^2/s$), and are not bound to a grid, but can freely move on the continuous landscape (i.e. we used an off-lattice model with double-precision floating-point format). At the beginning of a simulation, we randomly seed one founder bacteria of each strain onto the surface, free to grow and divide according to its life cycle.

In addition to the basic growth rate µ, the growth of a bacterium is influenced by its social behavior and its interaction with other community members. Costs, reducing growth, incur to individuals producing public goods, toxins and anti-toxins. Additional costs incur to sensitive cells taking up toxins. The uptake of a public good, meanwhile, has a positive effect on growth for the beneficiary. Accordingly, the growth of a wildtype public good producing strain (*W*), a policing strain producing a toxin together with an anti-toxin in addition to the public good (*P*), a non-public good producing toxin-sensitive cheating strain (*C*), and a toxin-resistant public good producing strain (*R*) are defined by the following set of functions:

$$G_W(t+1) = (\mu - c_{pg} + b\sum P_j)G_W(t) \quad (2)$$

$$G_P(t+1) = (\mu - c_{pg} - c_{tox} + b\sum P_j)G_P(t) \quad (3)$$

$$G_C(t+1) = (\mu + b\sum P_j)(1 - (\frac{\sum T_i}{\theta_T})^{\kappa})G_C(t) \quad (4)$$

$$G_R(t+1) = (\mu - c_{pg} - c_{res} + b\sum P_j)G_R(t) \quad (5)$$

where μ represents the basic growth rate. The value of μ was set to one for simplicity, and the increase in the cell radius was adjusted such that cell division occurs every 1,200 time steps, when neither public goods nor toxins are produced. The parameters c_{pg}, c_{tox}, c_{res} are the cost of public goods, toxin-anti-toxin, and anti-toxin production, respectively, whereby we assume that the toxin and the anti-toxin are equally costly (i.e. $c_{res} = c_{tox}/2$). While we set c_{pg} = 0.001 for each public good molecule produced, we varied c_{tox} in relation to c_{pg} to estimate the acceptable boundary costs of the policing mechanism. The term $\sum P_j$ stands for the number of public goods consumed by an individual and *b* for the benefit derived from this action. We set *b* = 0.01 per consumed public good molecule. Finally, the term $\sum T_i$ represents the number of consumed toxins. Toxins decrease the overall growth rate and lead to death when they accumulate beyond the threshold value θ_T. The negative effect toxins have on growth is further defined by the parameter κ, which we set to 3.5, resulting in a latency function, where the negative effects of toxins on growth accelerate with increased toxin uptake.

The public good producing strains constitutively secrete one molecule per time step, whereas the policing strain additionally secretes toxins at the same rate. The diffusion of the cells and molecules (described by the diffusion coefficients D_c, d_p and d_t, respectively) were modeled according to a Gaussian random walk, with a Gaussian random number generator based on the Box-Muller transform that converts uniformly distributed random numbers to normally distributed random numbers. Following diffusion, public goods and toxins were consumed whenever there was co-localization of molecules and cells. Both, public goods and toxins were represented by a point on the landscape up to the precision of the computer (double precision). The molecules remain in the simulation until they were either consumed or decayed. The probability of decaying was determined by a durability value δ (set to 500 time steps) and an exponential decay function

$$P_{decay} = 1 - e^{-\omega \Delta T/\delta} \tag{6}$$

where ΔT is the current lifespan of a molecule and $\omega = 0.1$ the steepness of the decay.

On our off-lattice landscape, individuals can physically overlap following cell growth and diffusion. To remove the overlaps, we implemented a procedure where cells are moved slightly apart from each other by a random factor scaled by a maximum pulling distance. In a final step, our stimulation involves a life-dead-control of each individual followed by the removal of dead cells. Cells can die because they are sensitive to toxins and have passed the threshold value θ_T for toxin uptake, or experience negative growth (i.e. they shrink) and fall below the minimal cell diameter of 0. Fig 1 depicts the order in which all the actions in our simulation were executed per time step. This simulation cycle continues until the total number of cells reaches the carrying capacity K = 1000 cells. For each of the simulated parameter settings, we performed 50 independent replicates. During the simulations, we monitored the

relative frequency of the individual strains. At the end of a simulation, we recorded the final strain frequency, calculated the mean time between two cell divisions, and the *per capita* public good and toxin uptake for each individual strain.

Simulating strain performance in monoculture

Policing in the form of toxin production generates additional costs for cooperators. We thus asked how large can c_{tox} be in relation to c_{pg}, in order to maintain a net benefit of cooperation. To address this question, we simulated the growth of *W*, *P*, and *C* in monoculture, and varied c_{pg}/c_{tox} from 4 to 0.25 in seven steps. A net benefit of cooperation is given when the policing strain *P* grows better than strain *C*, which neither produces public goods nor toxins. Because we know that the diffusion of cells and public goods influence the efficiency of cooperation [39], we repeated the simulations for three different diffusion regimes, including high (cell diffusion = 4 $\mu m^2/s$, molecule diffusion = 7.0 $\mu m^2/s$), medium (cell diffusion = 2.0 $\mu m^2/s$, molecule diffusion = 3.5 $\mu m^2/s$) and low (cell diffusion = 0.1 $\mu m^2/s$, molecule diffusion = 1.0 $\mu m^2/s$) diffusion. We then compared the time needed by the three strains to reach carrying capacity.

Simulating pairwise strain competitions

Next, we simulated competitions between the strains *W* and *C*, and the strains *P* and *C*. In both scenarios, *C* acts as a cheater and exploits the public good produced by either *W* or *P*. However, *C* is harmed by toxins in competition against *P*, but not when competing against *W*. While the competition between *W* and *C* provides information on the environmental conditions required for cooperation to be favored in the absence of policing, the competition between *P* and *C* addresses the question whether the range of conditions favoring cooperation

is extended with policing. In these simulations, we varied cell diffusion (0.0001 – 3.5 μm^2/s in steps of 0.5), public good diffusion (1 – 7 μm^2/s in steps of 1), toxin diffusion (1 – 7 μm^2/s in steps of 1), and the toxin death threshold θ_T (1000 - 2200 molecules in steps of 400).

Simulating three-way interactions

Finally, we asked whether policing is an evolutionary stable strategy or whether it can be outperformed by a public good producing strain *R* that no longer produces the toxin, but is resistant to it. To address this question, we simulated three-way interactions between and *W*, *C* and *R* (across a range of diffusion conditions: D_c = 1 – 2 μm^2/s in steps of 0.5, d_p = 3 – 5 μm^2/s in steps of 1). Because we do not necessarily expect an overall winner in these competitions, as strain frequency could potentially follow cyclical patterns [44], we extended our simulation code by implementing a random cell removal event, which is activated as soon as more than 30 % of the surface is covered with cells. This allowed us to keep population size at roughly *K*/2. In this set up, we followed strain frequency over time and arbitrarily stopped simulations after 80,000 time steps.

Acknowledgements

We thank Barbara König and Marta Manser for comments, the Services and Support for Science IT (S3IT) at the University of Zurich for technical support with the simulations on the ScienceCloud, and the Swiss National Science Foundation (grant no. PP00P3-139164) and the European Research Council (ERC-CoG grant no. 681295) for funding.

References

1. Sachs JL, Mueller UG, Wilcox TP, Bull JJ. The evolution of cooperation. Q Rev Biol. 2004; 79: 135-160.

2. West SA, Griffin AS, Gardner A. Evolutionary explanations for cooperation. Curr Biol. 2007; 17: R661-R672. doi: 10.1016/j.cub.2007.06.004.
3. Fehr E, Fischbacher U. The nature of human altruism. Nature. 2003; 425: 785- 791.
4. Clutton-Brock TH. Breeding together: kin selection and mutualism in cooperative vertebrates. Science. 2002; 296: 69-72.
5. Ratnieks FLW, Foster KR, Wenseleers T. Conflict resolution in insect societies. Annu Rev Entomol. 2006; 51: 581-608.
6. West SA, Diggle SP, Buckling A, Gardner A, Griffin AS. The social lives of microbes. Annu Rev Ecol Evol Syst. 2007; 38: 53-77. doi: 10.1146/annurev.ecolsys.38.091206.095740.
7. Nadell CD, Xavier JB, Foster KR. The sociobiology of biofilms. FEMS Microbiol Rev. 2009; 33: 206-224.
8. Ross-Gillespie A, Kümmerli R. Collective decision-making in microbes. Front Microbiol. 2014; 5: 54. doi: 10.3389/fmicb.2014.00054.
9. Cavaliere M, Feng S, Soyer OS, Jimenez JI. Cooperation in microbial communities and their biotechnological applications. Environ Microbiol. 2017; 19: 2949-2963. doi: 10.1111/1462-2920.13767.
10. Velicer GJ, Vos M. Sociobiology of the *Myxobacteria*. Annu Rev Microbiol. 2009; 63: 599-623. doi: 10.1146/annurev.micro.091208.073158.
11. Diggle SP, Griffin AS, Campell GS, West SA. Cooperation and conflict in quorum-sensing bacterial populations. Nature. 2007; 450: 411-414.
12. Sandoz KM, Mitzimberg SM, Schuster M. Social cheating in *Pseudomonas aeruginosa* quorum sensing. Proc Natl Acad Sci USA. 2007; 104: 15876-15881.
13. Griffin A, West SA, Buckling A. Cooperation and competition in pathogenic bacteria. Nature. 2004; 430: 1024-1027. doi: 10.1038/nature02744.
14. Kümmerli R, van den Berg P, Griffin AS, West SA, Gardner A. Repression of competition promotes cooperation: experimental evidence from bacteria. J Evol Biol. 2010; 23: 699-706. doi: 10.1111/j.1420-9101.2010.01936.x.
15. de Vargas Roditi L, Boyle KE, Xavier JB. Multilevel selection analysis of a microbial social trait. Mol Syst Biol. 2013; 9: 684. doi: 10.1038/msb.2013.42.
16. Ghoul M, Griffin AS, West SA. Toward and evolutionary definition of cheating. Evolution. 2014; 68: 318-331. doi: 10.1111/evo.12266.

17. Asfahl KL, Schuster M. Social interactions in bacterial cell-cell signaling. FEMS Microbiolol Ecol. 2017; 41: 92-107. doi: 10.1093/femsre/fuw038.

18. Özkaya Ö, Xavier KB, Dionisio F, Balbontin R. Maintenance of microbial cooperation mediated by public goods in single and multiple traits scenarios. J Bacteriol. 2017. doi: 10.1128/JB.00297-17.

19. van Gestel J, Weissing FJ, Kuipers OP, Kovács AT. Density of founder cells affects spatial pattern formation and cooperation in *Bacillus subtilis* biofilms. ISME J. 2014; 8: 2069-2079. doi: 10.1038/ismej.2014.52.

20. Kümmerli R, Griffin AS, West SA, Buckling A, Harrison F. Viscous medium promotes cooperation in the pathogenic bacterium *Pseudomonas aeruginosa*. Proc R Soc B. 2009; 276: 3531-3538. doi: 10.1098/rspb.2009.0861.

21. Mehdiabadi NJ, Jack CN, Farnham TT, Platt TG, Kalla SE, Shaulsky G, et al. Kin preferences in a social microbe. Nature. 2006; 442: 881-882.

22. Smukalla S, Caldara M, Pochet N, Beauvais A, Guadagnini S, Yan C, et al. *FLO1* Is a variable green beard gene that drives biofilm-like cooperation in budding yeast. Cell. 2008; 135: 726-737. doi: 10.1016/j.cell.2008.09.037.

23. Rendueles O, Zee PC, Dinkelacker I, Amherd M, Wielgoss S, Velicer GJ. Rapid and widespread de novo evolution of kin discrimination. Proc Natl Acad Sci USA. 2015; 112: 9076-9081. doi: 10.1073/pnas.1502251112.

24. Jousset A, Rochat L, Péchy-Tarr M, Keel C, Scheu S, Bonkowski M. Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters. ISME J. 2009; 3: 666-674. doi: 10.1038/ismej.2009.26.

25. Dandekar AA, Chugani S, Greenberg EP. Bacterial quorum sensing and metabolic incentives to cooperate. Science. 2012; 338: 264-266. doi: 10.1126/science.1227289.

26. Ross-Gillespie A, Dumas Z, Kümmerli R. Evolutionary dynamics of interlinked public goods traits: an experimental study of siderophore production in *Pseudomonas aeruginosa*. J Evol Biol. 2015; 28: 29-39. doi: 10.1111/jeb.12559.

27. Mitri S, Foster KR. Pleiotropy and the low cost of individual traits promote cooperation. Evolution. 2016; 70: 488-494. doi: 10.1111/evo.12851.

28. Popat R, Harrison F, da Silva AC, Easton SA, McNally L, Williams P, et al. Environmental modification via a quorum sensing molecule influences the social

landscape of siderophore production. Proc R Soc B. 2017; 284. doi: 10.1098/rspb.2017.0200.

29. Kümmerli R, Brown SP. Molecular and regulatory properties of a public good shape the evolution of cooperation. Proc Natl Acad Sci USA. 2010; 107: 18921-18926. doi: 10.1073/pnas.1011154107.

30. Xavier JB, Kim W, Foster KR. A molecular mechanism that stabilizes cooperative secretions in *Pseudomonas aeruginosa*. Mol Microbiol. 2011; 79: 166-179. doi: 10.1111/j.1365-2958.2010.07436.x.

31. Sexton DJ, Schuster M. Nutrient limitation determines the fitness of cheaters in bacterial siderophore cooperation. Nat Commun. 2017; 8: 230. doi: 10.1038/s41467-017-00222-2.

32. Manhes P, Velicer GJ. Experimental evolution of selfish policing in social bacteria. Proc Natl Acad Sci USA. 2011; 108: 5357-5362. doi: 10.1073/pnas.1014695108.

33. Inglis RF, West S, Buckling A. An experimental study of strong reciprocity in bacteria. Biol Lett. 2014; 10: 20131069. doi: 10.1098/rsbl.2013.1069.

34. Wang M, Schaefer AL, Dandekar AA, Greenberg EP. Quorum sensing and policing of *Pseudomonas aeruginosa* social cheaters. Proc Natl Acad Sci USA. 2015; 112: 201500704. doi: 10.1073/pnas.1500704112.

35. Majerczyk C, Schneider E, Greenberg EP. Quorum sensing control of Type VI secretion factors restricts the proliferation of quorum-sensing mutants. eLife. 2016; 5: e14712. doi: 10.7554/eLife.14712.

36. Evans KC, Benomar S, Camuy-Vélez LA, Nasseri EB, Wang X, Neuenswander B, et al. Quorum-sensing control of antibiotic resistance stabilizes cooperation in *Chromobacterium violaceum*. ISME J. 2018; in press. doi: 10.1038/s41396-018-0047-7.

37. Driscoll WW, Pepper JW. Theory for the evolution of diffusible external goods. Evolution. 2010; 64: 2682-2687. doi: 10.1111/j.1558-5646.2010.01002.x.

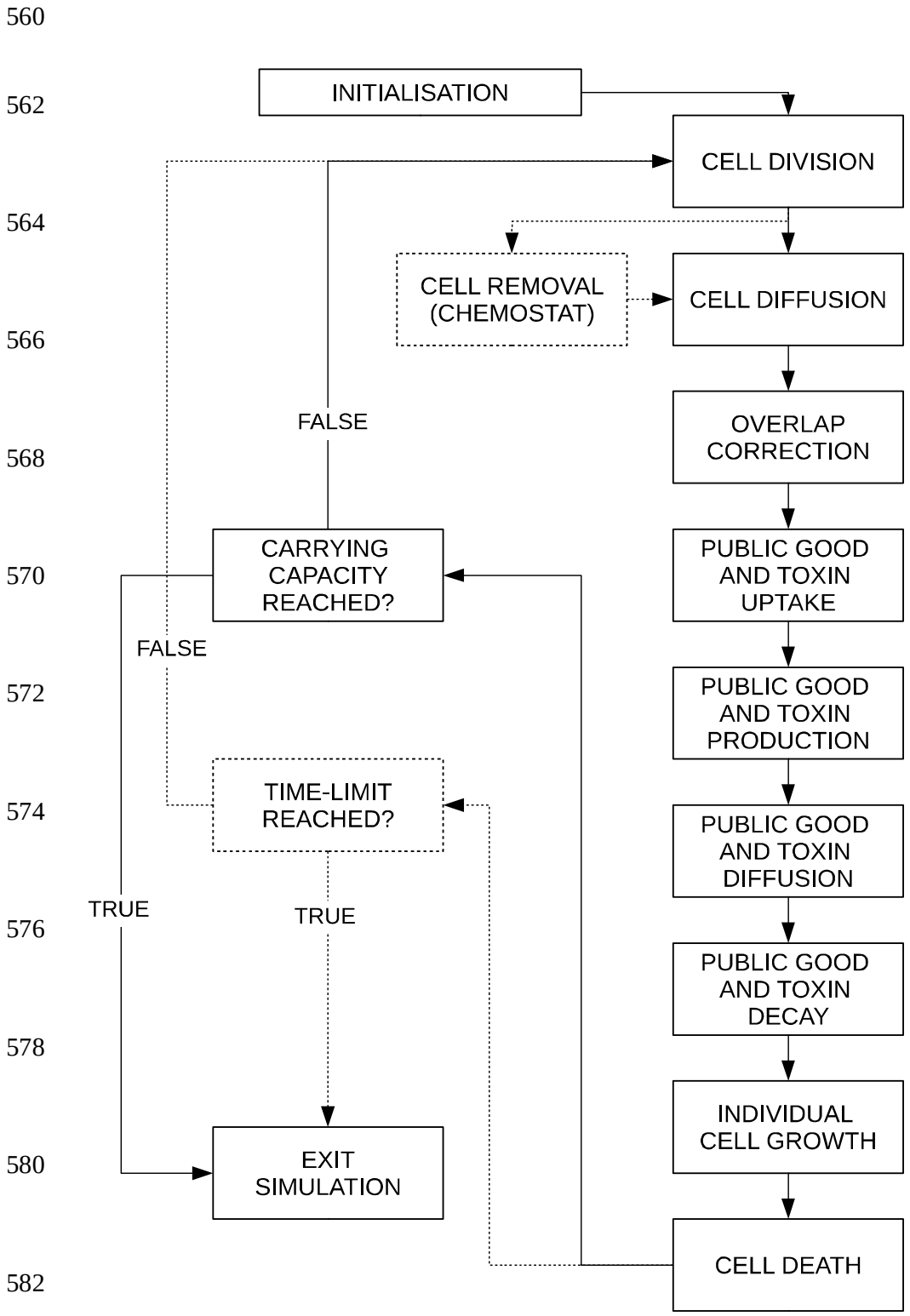
38. Inglis RF, Roberts PG, Gardner A, Buckling A. Spite and the scale of competition in *Pseudomonas aeruginosa*. Am Nat. 2011; 178: 276-285. doi: 10.1086/660827.

39. Dobay A, Bagheri HC, Messina A, Kümmerli R, Rankin DJ. Interaction effects of cell diffusion, cell density and public goods properties on the evolution of cooperation in digital microbes. J Evol Biol. 2014; 27: 1869-1877. doi: 10.1111/jeb.12437.

40. Boyd R, Gintis H, Bowles S, Richerson PJ. The evolution of altruistic punishment. Proc Natl Acad Sci USA. 2003; 100: 3531-3535.
41. Monod J. The growth of bacterial cultures. Annu Rev Microbiol. 1949; 3: 371-394.
42. Julou T, Mora T, Guillon L, Croquette V, Schalk IJ, Bensimon D, et al. Cell-cell contacts confine public goods diffusion inside *Pseudomonas aeruginosa* clonal microcolonies. Proc Natl Acad Sci USA. 2013; 110: 12577-12582. doi: 10.1073/pnas.1301428110.
43. Weigert M, Kümmerli R. The physical boundaries of public goods cooperation between surface-attached bacterial cells. Proceedings of the Royal Society B: Biological Sciences. 2017; 284: 20170631. doi: 10.1098/rspb.2017.0631.
44. Kerr B, Riley MA, Feldman MW, Bohannan BJM. Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. Nature. 2002; 418: 171-174. doi: 10.1038/nature00823.
45. Kelsic ED, Zhao J, Vetsigian K, Kishony R. Counteraction of antibiotic production and degradation stabilizes microbial communities. Nature. 2015; 521: 516-519. doi: 10.1038/nature14485.
46. Inglis RF, Biernaskie JM, Gardner A, Kümmerli R. Presence of a loner strain maintains cooperation and diversity in well-mixed bacterial communities. Proc R Soc B. 2016; 283: 20152682.
47. Frank SA. Mutual policing and repression of competition in the evolution of cooperative groups. Nature. 1995; 377: 520-522.
48. Clutton-Brock TH, Parker GA. Punishment in animal societies. Nature. 1995; 373: 209-216.
49. Frank SA. Perspective: repression of competition and the evolution of cooperation. Evolution. 2003; 57: 693-705.
50. Ratnieks FLW, Wenseleers T. Altruism in insect societies and beyond: voluntary or enforced? Trends Ecol Evol. 2008; 23: 45-52.
51. Kümmerli R. A test of evolutionary policing theory with data from human societies. PLoS One. 2011; 6: e24350. doi: 10.1371/journal.pone.0024350.
52. Raihani NJ, Thornton A, Bshary R. Punishment and cooperation in nature. Trends Ecol Evol. 2012; 27: 288-295. doi: 10.1016/j.tree.2011.12.004.

53. Cant MA, Nichols HJ, Johnstone RA, Hodge SJ. Policing of reproduction by hidden threats in a cooperative mammal. Proc Natl Acad Sci USA. 2013; 111: 326-330. doi: 10.1073/pnas.1312626111.
54. Mitri S, Foster KR. The genotypic view of social interactions in microbial communities. Annu Rev Genet. 2013; 47: 247-273. doi: 10.1146/annurev-genet-111212-133307.
55. West SA, Griffin AS, Gardner A. Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. J Evol Biol. 2007; 20: 415-432. doi: 10.1111/j.1420-9101.2006.01258.x.
56. Bernier SP, Workentine ML, Li X, Magarvey NA, O'Toole GA, Surette MG. Cyanide toxicity to *Burkholderia cenocepacia* is modulated by polymicrobial communities and environmental factors. Front Microbiol. 2016; 7: 1-14. doi: 10.3389/fmicb.2016.00725.
57. Darch SE, West SA, Winzer K, Diggle SP. Density-dependent fitness benefits in quorum-sensing bacterial populations. Proc Natl Acad Sci USA. 2012; 109: 8259-8263. doi: 10.1073/pnas.1118131109.
58. Foster K. The sociobiology of molecular systems. Nat Rev Genet. 2011; 12: 193-203. doi: 10.1038/nrg2903.
59. Kiers ET, Rousseau RA, West SA, Denison RF. Host sanctions and the legume-rhizobium mutualism. Nature. 2003; 425: 78-81.
60. Wenseleers T, Ratnieks FLW. Enforced altruism in insect societies. Nature. 2006; 444: 50.

Figure 1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INITIALISATION
CELL DIVISION
CELL REMOVAL (CHEMOSTAT)
CELL DIFFUSION
OVERLAP CORRECTION
PUBLIC GOOD AND TOXIN UPTAKE
PUBLIC GOOD AND TOXIN PRODUCTION
PUBLIC GOOD AND TOXIN DIFFUSION
PUBLIC GOOD AND TOXIN DECAY
INDIVIDUAL CELL GROWTH
CELL DEATH
CARRYING CAPACITY REACHED?
TIME-LIMIT REACHED?
EXIT SIMULATION
FALSE
FALSE
TRUE
TRUE

Fig 1. Flow diagram of the computer simulation showing the order of each procedure called during a single time step. Our platform allows for two different types of culturing conditions: batch-culture growth, where simulations stop when populations reach the carrying capacity $K = 1000$ (solid lines); and continuous growth in a chemostat, where simulations stop after a given time period (dashed lines). To guarantee continuous growth, the chemotstat cycle comprised a random cell removal mechanism, such that population size was kept constant at $K/2$. Each box describes a key element in a cell's life cycle, affecting its fitness: i.e. cells grew according their fitness equations (see main text) and divided when reaching the threshold radius of 1 μm (two times the initial radius). Since cells are free to move on a continuous landscape, they can overlap following diffusion. To remove cell overlap, we used a procedure based on the physics of hard-core interactions used in molecular dynamics and cell elasticity.

Figure 2

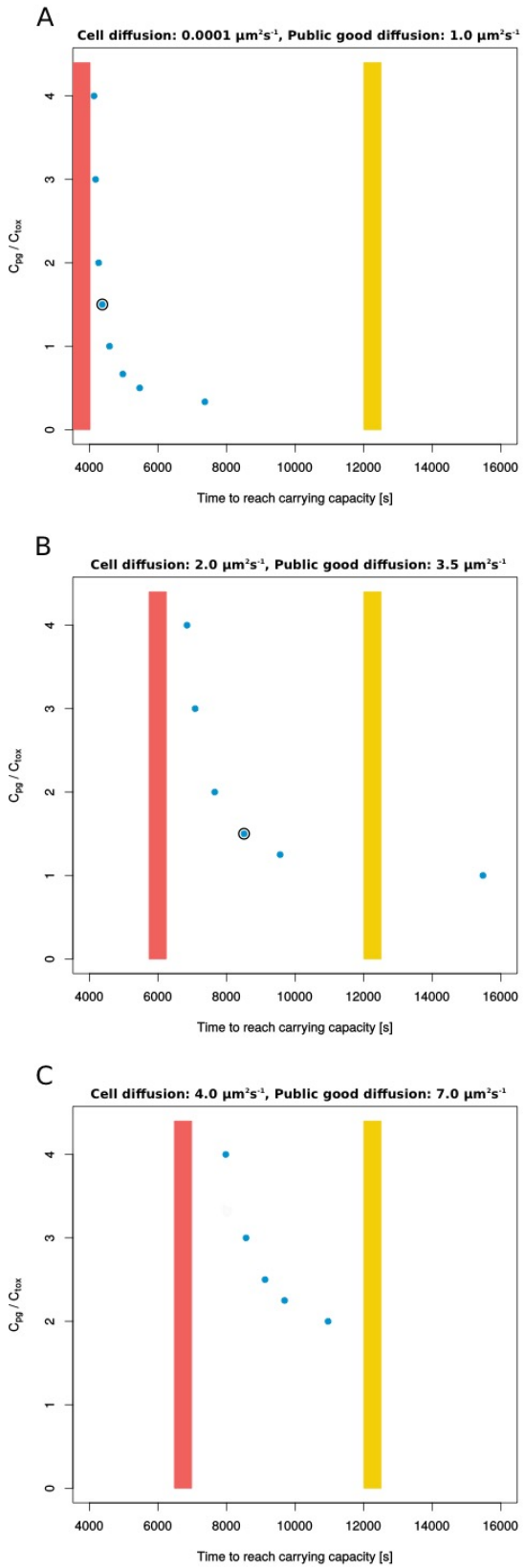
A
Cell diffusion: 0.0001 μm²s⁻¹, Public good diffusion: 1.0 μm²s⁻¹
Cpg / Ctox
Time to reach carrying capacity [s]
B
Cell diffusion: 2.0 μm²s⁻¹, Public good diffusion: 3.5 μm²s⁻¹
Cpg / Ctox
Time to reach carrying capacity [s]
C
Cell diffusion: 4.0 μm²s⁻¹, Public good diffusion: 7.0 μm²s⁻¹
Cpg / Ctox
Time to reach carrying capacity [s]

Fig 2. Growth in monoculture and the relative cost of policing. We examined how large the cost of policing c_{tox} can be, relative to the cost of public good production c_{pg}, to guarantee a net benefit of cooperation. To address this question, we compared the growth of the policing strain (producing toxins and public goods) with the growth of the cooperator strain (producing only public goods) and the cheater strain (producing neither toxins nor public goods) in monoculture for a range of c_{pg}/c_{tox} ratios, under three different diffusion regimes. (A) environment with low diffusivity (cell diffusion = 0.0001 $\mu m^2/s$, molecule diffusion = 1.0 $\mu m^2/s$), (B) environment with intermediate diffusivity (cell diffusion = 2.0 $\mu m^2/s$, molecule diffusion = 3.5 $\mu m^2/s$) and (C) environment with high diffusivity (cell diffusion = 4 $\mu m^2/s$, molecule diffusion = 7.0 $\mu m^2/s$). The red and yellow bars indicate the time needed by the cooperator and the cheater strain to reach carrying capacity, respectively. For policing to evolve, the time needed by the policer strain (blue dots) to reach carrying capacity must be within these boundaries. Values represent averages of 50 independent simulations. We set c_{pg}= 0.001, while varying c_{tox} from 0.00025 to 0.004. The blue dots marked with a black circle correspond to c_{pg}/c_{tox}= 1.5, the relative policing cost used for all subsequent simulations.

Figure 3

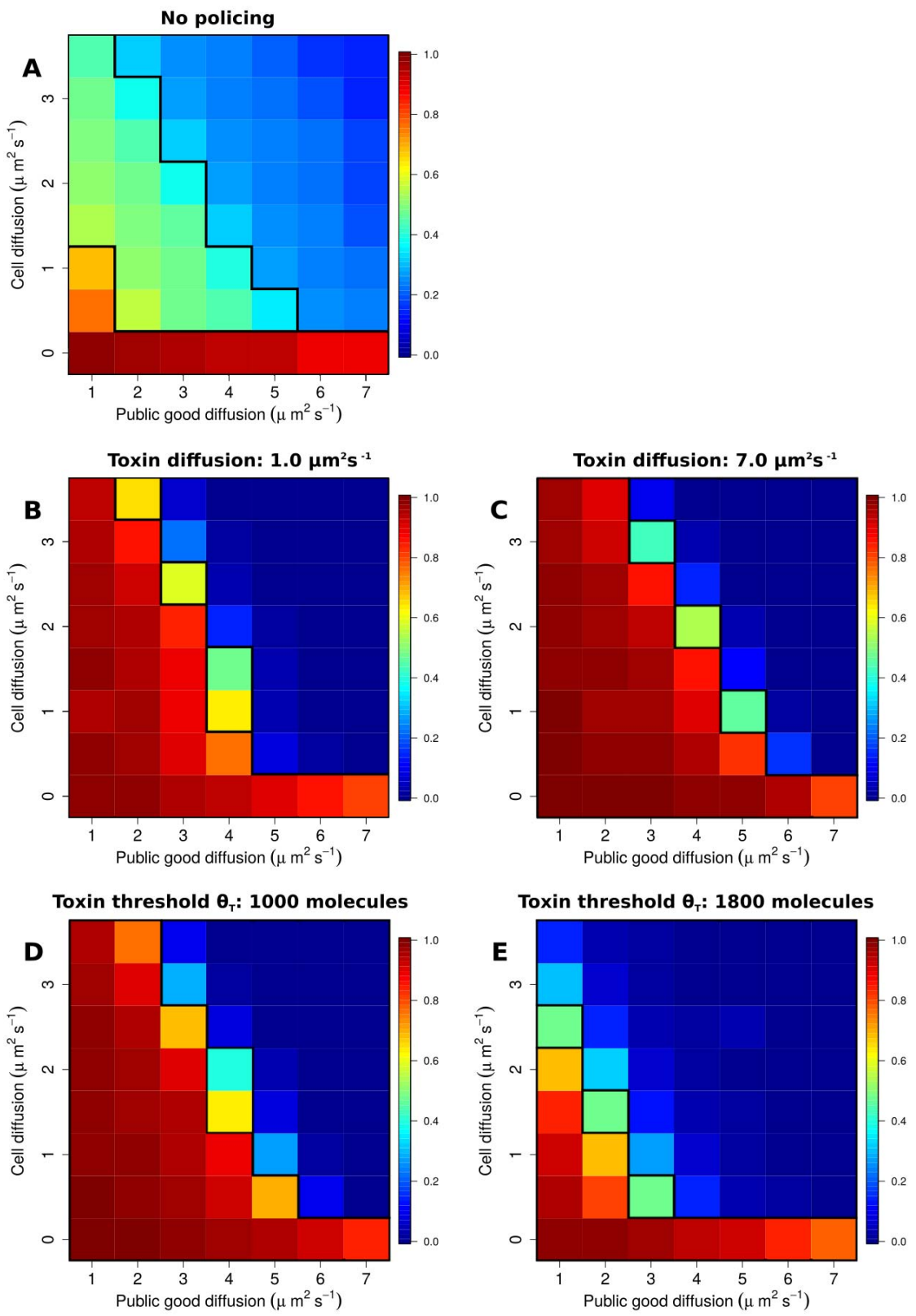
No policing
A
Toxin diffusion: 1.0 μm²s⁻¹
B
Toxin diffusion: 7.0 μm²s⁻¹
C
Toxin threshold θ_T: 1000 molecules
D
Toxin threshold θ_T: 1800 molecules
E
Cell diffusion (μ m² s⁻¹)
Public good diffusion (μ m² s⁻¹)
0
1
2
3
4
5
6
7
0.0
0.2
0.4
0.6
0.8
1.0

Fig 3. Competitions between cheaters and different types of cooperators. Heat maps show the frequency of the non-policing (A) and the policing (B-E) cooperator strain in competition with the cheater strain, at the time point where communities reached carrying capacity. (A) Outcomes of competitions between the cheater strain and the non-policing cooperator strain (producing public goods but no toxins) across a range of cell diffusion (0.0001 - 3.5 $\mu m^2/s$) and public good diffusion (1.0 - 7.0 $\mu m^2/s$) values. (B-E) Outcomes of competitions between the cheater strain and the policing cooperator strain (producing toxins in addition to public goods) across the same range of cell and public good diffusion values. Specific scenarios include cases of: (B) low toxin diffusion (d_t= 1.0 $\mu m^2/s$ and θ_T= 1000); (C) high toxin diffusion (d_t= 7.0 $\mu m^2/s$ and θ_T= 1000); (D) high toxin efficiency (θ_T= 1000 and d_t= 4.0 $\mu m^2/s$); and (E) intermediate toxin efficiency (θ_T= 1800 and d_t= 4.0 $\mu m^2/s$). The black lines delineate the three regions, where cooperators were either favored (frequency f of cooperators > 2/3), disfavored (f < 2/3) or could coexist with cheaters (1/3 < f < 2/3). Values represent averages of 50 independent simulations.

Figure 4

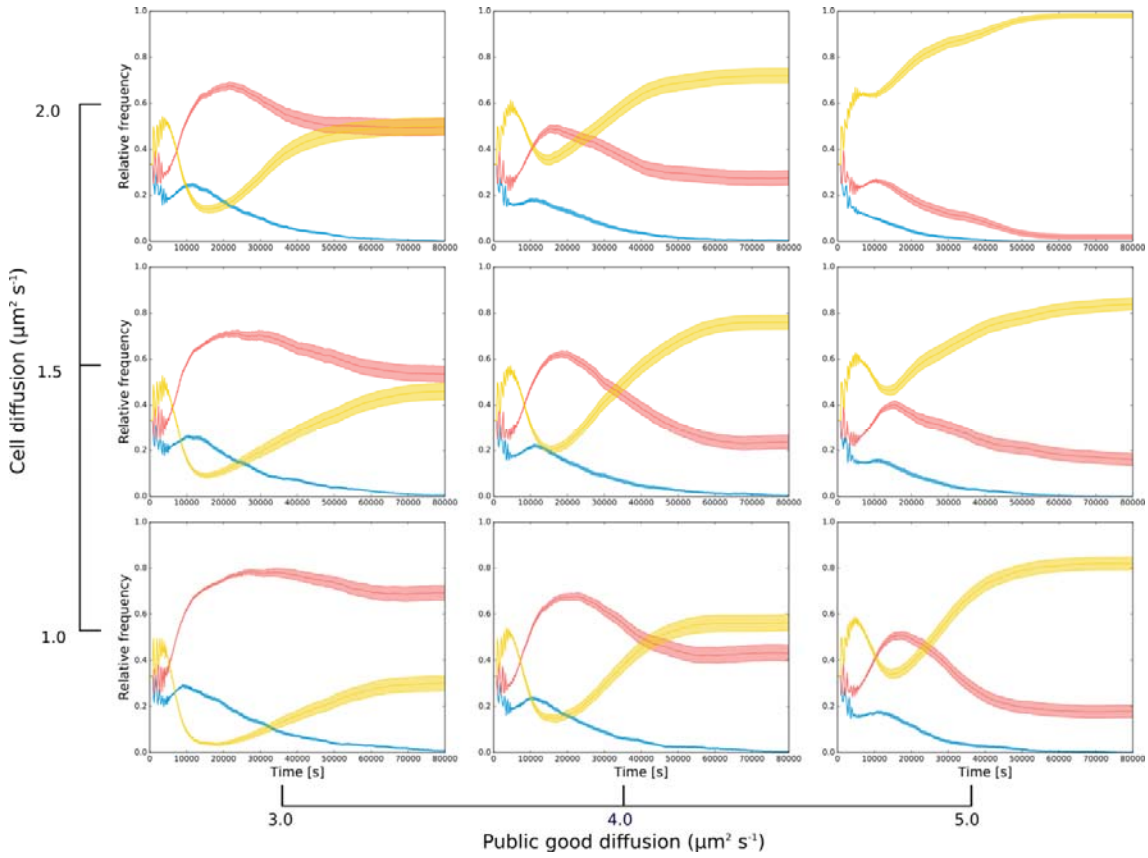
Cell diffusion (μm² s⁻¹)
2.0
1.5
1.0
Relative frequency
Time [s]
3.0
4.0
5.0
Public good diffusion (μm² s⁻¹)

Fig 4. Policing is selected against when the genetic linkage between public good and toxin production breaks. We examined whether policing is an evolutionary stable strategy (ESS) or whether it is outperformed by a public-good producing mutant strain that no longer invests in the toxin, but is resistant to it. We assume that such a mutant can directly evolve from the policing strain when the genetic linkage between public good and toxin production breaks. To address this question, we simulated three-way interactions between cheaters (yellow lines), cooperating policers (blue lines), and toxin-resistant non-policing cooperators (red lines) across a range of public good and cell diffusion values. Our simulations in a continuous culture show that policing is selected against under all conditions, demonstrating that policing is a second-order public good that is exploitable by non-policing cooperators. Values depict means of 50 independent replicates with the standard error of the mean (transparent areas). For all simulations, the toxin diffusion and efficiency were kept constant at $d_t = 7.0$ μm^2/s and $\theta_T = 1000$, respectively.

Supplementary Figure 1

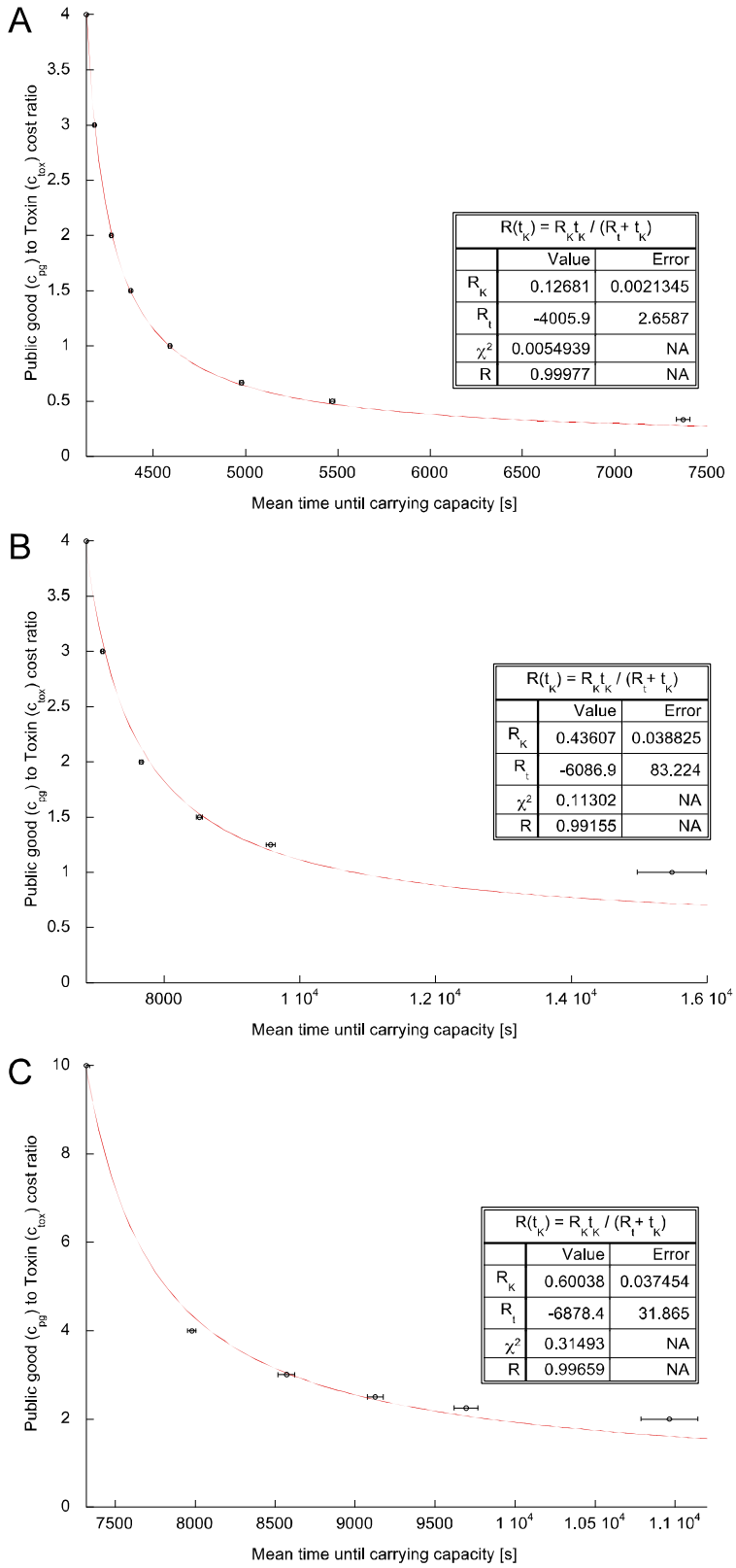

Fig S1. Monod's equation explains the relationship between the relative cost of policing and strain fitness. We simulated the growth of the policing strain (producing a toxin and a public good) in monoculture for a range of c_{pg}/c_{tox} ratios, under three different diffusion regimes. We kept the cost of public good production constant at c_{pg} = 0.001, while varying the cost of toxin production c_{tox} from 0.00025 to 0.004. The fitness of the policing strain (measured as the time required to reach carrying capacity) declined with higher relative costs of toxin production. The decline was adequately captured by Monod's equation under conditions of (A) low diffusion (coefficient of correlation R = 0.999); (B) medium diffusion (R = 0.991); and (C) high diffusion (R = 0.997). Details on diffusion parameter values can be obtained from Fig 2. Open circles show means of 50 independent replicates with the standard error of the mean.