Observation with microcalorimetry: behaviour of *P. aeruginosa* in mixed cultures with *S. aureus* and *E. coli*

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Abstract

Bacteria exist as mixed cultures of different species in their natural environments. However, most laboratory characterization experiments, for instance, susceptibility testings are routinely conducted on pure species of bacteria. Interactions that may naturally occur and their consequent effects on the metabolisms of the individual species are missed by this approach. In this work, we explored the potential of isothermal microcalorimetry (IMC), a technique which has been established to characterize microorganisms to study the behaviour of mixed cultures of *Pseudomonas aeruginosa* with *Staphylococcus aureus* and *Escherichia coli*. The species were firstly studied as pure culture at 10^6 CFU/mL densities in nutrient broth (NB). The species were then introduced into NB to give an equivalent inoculum of 10^6 CFU/mL of each. Additionally, the inoculum density of one species was maintained at 10^6 CFU/mL and the other varied between 10^3-10^5 CFU/mL. The IMC power-time curves were characteristic for the species in the medium. The total heat output (Q_t), amplitude of first peak (P_1), time of registration of first peak (t_1), final metabolic/maximum peak (P_{fmax}) and time of registration of the final metabolic/maximum peak (t_{fmax}) were calculated from the power-time data. A mixed culture of *P. aeruginosa* and *S. aureus* at equal densities exhibited metabolism synonymous to *P. aeruginosa* alone. When the density of *P. aeruginosa* was decreased, *S. aureus* gradually recovered showing power-time profiles that demonstrated this. The plate count data collected at the end of IMC experiments corroborated with the observation in the IMC. Mixed cultures of *P. aeruginosa* and *E. coli* at equal densities showed power-time profiles representative of both species demonstrating the co-dominance of the organisms. However the plate counts data showed *P. aeruginosa* was inhibited in growth.

Keywords: *P. aeruginosa*, *S. aureus*, *E. coli*, mixed culture, interspecies interaction, isothermal microcalorimetry

1. Introduction

Traditionally, *in vitro* microbial characterization, particularly, susceptibility testings are routinely conducted in pure culture. While these tests are useful, giving important insight on how a specific organism or species survives or adapts to a myriad of variables, the scenarios created may not accurately be representative of real-life situations. For example, the many infections in humans and animals, which occur as associations of two or more bacterial species, are not truly represented by these tests (Gilligan, 1991; Goldstein and Snydman, 2004). In isolation, a pure culture may behave much differently than when it is combined with other species. Within a community, it is expected that the different species will interact with each other with a resultant effect, which may differ from the effect of the individual component species (Filkins et al., 2015; Rueger et al., 2012). This could have consequences on response of the organism to a treatment, for instance, the efficacy of a selected therapeutic.

One main challenge is the lack of simple, fast, high throughput and inexpensive assays for the routine conduct of mixed species characterization. The constraints with traditional microbiological techniques (based on selective or differential spread plate methods) make it difficult for the routine performance of such assays to inform real-time interspecies relationships. The advent of molecular tools such as denaturing gradient gel electrophoresis (DGGE; Nakatsu, 2007), randomly amplified polymorphic DNA (RAPD; Erlandson and Batt, 1997), real-time polymerase chain reaction (PCR), terminal restriction fragment length polymorphism analysis (T-RFLP; Kitts, 2001), and length heterogeneity (LH)-PCR (Ritchie et al., 2000) which do not require culture for analysis have enabled the characterization of the functional diversity of mixed microbial samples. However, they have not been particularly useful for species-specific enumeration of mixed cultures, which is important for understanding of microbial interactions and the mechanisms underlying such

relational dynamics. Nonetheless, quantitative PCR (qPCR; Grattepanche et al., 2005), quantitative T-RFLP (qT-RFLP; Schmidt et al., 2007, Schmidt et al., 2011) and fluorescent in situ hybridization (FISH; DuTeau et al.,1998) have been successfully employed for quantitative analysis of mixed cultures, providing useful information about interactions between the different species of bacteria. These advanced techniques can however be expensive, require some expertise, reagents and the pretreatment of the culture before analysis. This warrants exploring other methods.

Isothermal microcalorimetry (IMC) is a technique, which measures the heat flow of chemical, physical and biological processes. It has been reported to circumvent some of the limitations of traditional microbiologicial assay techniques and been vastly applied in microbial characterization (Beezer, 1980). For instance, it has been used for monitoring and quantification of soil microbial activity and contamination (Bravo et al., 2011; Guo et al., 2012); detection of infection and contamination of clinical products and samples (Trampuz et al., 2007); determination of effects of antimicrobial compounds (Gaisford et al., 2009; O'Neill et al., 2003) including the mode of action of antimicrobial compounds-bacteriostatic or bacteriocidal (von Ah et al., 2008), viral infections and activities of antiviral compounds (Heng et al., 2005; Tan and Lu, 1999); spoilage of food (Alklint et al., 2005) amongst others. However, with all the microbiological applications, only few studies have been conducted on mixed culture with the aim of differential detection or to investigate the relationship between two or more bacteria (Fredua-Agyeman et al., 2017).

In this study, we explored the potential of IMC to investigate the growth and behaviour of *Pseudomonas aeruginosa* in mixed culture with *Staphylococcus aureus* (which present as mixed infection in lung and chronic wound infections; Bessa et al., 2015) and with *Escherichia coli* (which occur in urinary tract infections).

2. Materials and methods

Staphylococcus aureus NCIMB 9518 and *Pseudomonas aeruginosa* NCIMB 8628 were obtained from ConvaTec Ltd. *Escherichia coli* ATCC 25922 was purchased from American Type Culture Collection, USA. The strains were grown in Nutrient broth (NB, Oxoid) in a shaking incubator (Innova 4080, New Brunswick Scientific, UK) at 37^{o}C. The cells were harvested, washed in phosphate buffered saline (PBS) and resuspended in 15% v/v glycerol in ¼ th strength Ringer's solution to a density of 10^{8} CFU/mL when they reached the stationary phase of growth. The resuspended cells were frozen in 1.8 mL aliquots in vials over liquid nitrogen and stored in liquid nitrogen until required (Beezer et al., 1976).

A vial was removed from the storage liquid nitrogen container, thawed at 40^{o}C for 3 min and vortexed for 1 min prior to use. 30 µL of the thawed culture of respective species was inoculated into 2970 µL of pre-warmed NB in a sterile 3 mL calorimetric glass ampoule giving a density of 10^{6} CFU/mL for pure culture experiments. For mixed culture experiments, one species was inoculated at a constant density of 10^{6} CFU/mL and mixed with different densities of the other (10^{3}-10^{6} CFU/mL) in a 3 mL calorimetric ampoule. The ampoules were sealed with crimped caps and vortexed for 10 s. They were placed in the thermal equilibration position of a Thermometric Thermal Activity Monitor 2277 (TAM 2277, TA Instruments Ltd., UK) set at 37^{o}C (±0.1^{o}C). The loaded ampoules were allowed to equilibrate at this intermediate position for 30 min before being lowered into the measurement position. Data were captured with Digitam 4.1 every 10 s with an amplifier range of 1000 µW until the power-time data returned to baseline. Data were analysed with Origin Pro 8.6 (Microcal Software Inc.). The reference ampoule was loaded with 3 mL of sterile media. The instrument was calibrated at regular intervals.

Relative growths of the species were determined after microcalorimetric measurements by plating 50 µL of serially diluted cultures on Cetrimide agar (Oxoid, Basingstoke, UK), Mannitol salt agar (Oxoid, Basingstoke, UK) and MacConkey agar (Oxoid, Basingstoke, UK). pH measurements (pHenomenal®, UK) were also done post calorimetric experiments. All procedures were carried out aseptically.

3. Results and Discussion

IMC records power (µW or μJs^{-1}) as a function of time (t). The growth of microorganisms in the microcalorimeter typically results in an exponentially increasing signal (representative of the heat produced by the growing microorganism) until the concentration of the energy source becomes limiting, and there is a build up of toxic metabolites, in which case the power signal approaches baseline. The data in Fig. 1, which compares the power-time growth curves of *P. aeruginosa*, *S. aureus* and their mixed culture at equal densities illustrate this. The power-time curve of *P. aeruginosa* shows increasing signal with two metabolic peaks; the second with greater energy and without significant lag after the first. *S. aureus* shows initial peak as *P. aeruginosa* but shows another metabolic phase, followed by a long period of latency then a final metabolic peak of intensity greater than the first two metabolic phases. The main difference between the profile of *P. aeruginosa* and *S. aureus* is the duration and metabolic activity preceding the final exponential metabolic phase.

To express the data in a more quantitative way, the total heat output (Q_t) which is the total area under growth curve (AUC), amplitude of first peak (P_1), time of registration of first peak (t_1), final metabolic/maximum peak (P_{fmax}) and time of registration of the final metabolic/maximum peak (t_{fmax}) were calculated from the power-time data and this shows significant differences ($p < 0.05$) in Q_t, P_1, t_1 and t_{fmax} between the two

species (Table 1). The growth profile of mixed cultures of the two species shows an initial metabolic peak and a second of greater intensity. There is insignificant lag between the two peaks but a slight metabolic activity can be seen in the curve prior to the final exponential phase. The power-time data for the mixed culture shows significant differences ($p < 0.05$) in P_1 and t_{fmax} between the mixed culture and pure *S. aureus* but no significant differences was seen between the former and pure *P. aeruginosa*. Overall, when the duration and metabolic phases are considered, the power-time profile of the mixed culture shows more similarities to *P. aeruginosa* than *S. aureus.* These factors make one reason that perhaps the growth of *P. aeruginosa* dominated in the mixed culture although it can also be argued that an interaction occurred between the two species.

The mixed cultures of *P. aeruginosa* and *S. aureus* at different densities of the two are compared in Fig. 2. The profile of the mixed cultures appear to revert from the dominating characteristic profile of *P. aeruginosa* as the density of *P. aeruginosa* is decreased to curves that show prominence of *S. aureus*. The derivatives from the power-time data support this observation. Although not very reflective in the Q_t values, the P_1 and t_1 values show significant differences ($p < 0.05$) between pure *S. aureus* and its mixed cultures with *P. aeruginosa* at higher densities of *P. aeruginosa.* Pure *S. aureus* shows significant differences ($p < 0.05$) in t_{fmax} values between all its mixed cultures with *P. aeruginosa* whilst pure *P. aeruginosa* on the otherhand shows no significant differences in Q_t values between all mixtures with *S. aureus*. However it shows significant differences ($p < 0.05$) in P_1, t_1 and t_{fmax} values when its density is smaller relative to *S. aureus* in the mixed cultures. The data may imply some interaction between the two species in mixed cultures. The data also suggest that *P. aeruginosa* dominated at low densities of *S. aureus* while *S. aureus* dominated at lower densities of *P. aeruginosa*. However, overall, the profiles of the mixed cultures share more similarities to that of *P. aeruginosa*. The pH measurement

and viable cell count determination after microcalorimetric experiments are given in Table 2. The metabolism of *S. aureus* showed more acidic waste than that of *P. aeruginosa*. The plate count data showed that *S. aureus* was inhibited in growth especially when its inoculum density was lower than that of *P. aeruginosa* in the mixed cultures.

The growth curves of *P. aeruginosa* and *E. coli* are shown and compared in Fig. 3. The growth curve of *E. coli* shows multiple exponential phases and major differences between *P. aeruginosa*. *E. coli* has mixed-acid fermentation. It is plausible that a sequential degradation and uptake of the carbon sources present in the medium (given the limited oxygen in the ampoule) could have contributed to the detailed structure of the growth curve of *E. coli* (Schaarschmidt and Lamprecht, 1978). Mixed culture of both species show some characteristic metabolic peaks of *E. coli* within the first 3 hours but subsequent peak after that could be identified with *P. aeruginosa*. The Q_t, P_1, t_1, P_{fmax} and t_{fmax} values of the pure species and the mixed cultures are shown in Table 3. There are significant differences ($p < 0.05$) in Q_t, P_1, P_{fmax} and t_{fmax} between the two pure species. Although the time of registration of the first peak does not significantly differ between the two species, *E. coli* has a P_1 value which is larger than *P. aeruginosa* and even larger than its P_{fmax} value unlike in the case of *P. aeruginosa* and *S. aureus*. The mixed culture at equal density of *P. aeruginosa* and *E. coli* shows no significant difference to the pure species in the Q_t value but significant difference to *P. aeruginosa* in the P_1 value and to *E. coli* in the P_{fmax} and t_{fmax} values. The data from the pure species and mixed culture make one reason that possibly an interaction occurred between the two species in mixed culture. When densities of the species in mixed culture were decreased relative to the other, growth profiles of the mixed cultures began to appear like the profiles of the pure species (Fig. 4). *P. aeruginosa* showed significant differences ($p < 0.05$) in P_1 value to the mixed cultures when its density was lesser than that of *E. coli* in the mixed culture. It

however showed differences in both P_{fmax} and t_{fmax} values when its density in the mixed culture was lowest (10^3 CFU/mL). *E. coli* showed significant differences ($p < 0.05$) to the mixed culture in P_1, t_1, P_{fmax} and t_{fmax} values when its density in the mixed culture was 10^4 CFU/mL or less. One could infer that the growth profile of *P. aeruginosa* was particularly dominant in the mixed culture when the density of *E. coli* was between 10^4 to 10^3 CFU/mL; *E. coli* could have dominated when the density of *P. aeruginosa* in the mixed culture was between 10^5 to 10^3 CFU/mL. The pH measurement (Table 4) shows that *E. coli* produced acidic waste, which could have accounted for part of its dominance. Plate counts after microcalorimetric measurements (Table 4) show that *P. aeruginosa* was inhibited in the mixed culture particularly when its inoculum density was between 10^5 to 10^3 CFU/mL.

This study has shown that *P. aeruginosa*, *S. aureus* and *E. coli* have characteristic growth curves in NB. When mixed in different densities, it was observed that dominance of growth of one species occurred over the other. The observed dominance in the mixed cultures could be due to adaptation and effective use of nutrients in the medium or the production of inhibitory substances that could have affected the growth of the other species. For mixtures with *E. coli*, these inhibitory substances could be acidic waste. But it is also likely specific inhibitory exo-metabolite could have played a role. Both the Gram-negative species: *P. aeruginosa* and *E. coli* are known to produce allelopathic substances that target and kill other microorganisms (Gordon and O'Brien, 2006; Michel-Briand and Baysse, 2002). *P. aeruginosa* in particular is known to produce antistaphylococcal substances: 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), hydrogen cyanide and pyocyanin that suppress the growth of *S. aureus* and many other bacteria (Biswas et al., 2009; Castric, 1975; Hassan and Fridovich, 1980; Machan et al., 1992). Inhibition of *S. aureus* by *P. aeruginosa* has been reported by a number of authors (Baldan et al., 2014; Biswas et al., 2009; Filkins et al., 2015; Hoffman et al., 2006; Perestelo et al.,

1985) and has been associated with the fact that in co-infections with both species, *S. aureus* is frequently less cultured although both could also be co-isolated in infected cultures (Hoffman et al., 2006). The predominance has been associated with compounds produced by *P. aeruginosa* in mixed cultures, which inhibits *S. aureus*. It has also been previously demonstrated that production of inhibitory compounds by *P. aeruginosa*, particularly pyocyanin (which targets the electron transport chain of *S. aureus*) coincides exactly with the time *S. aureus* concentration decreases in co-cultures when both were previously growing parallel together for approximately 8 h (Biwas et al., 2009). Using IMC, this inhibition has also been observed. Although growth of *S. aureus* is characterisitic and occurs beyond 8 h, a mixed culture with *P. aeruginosa* at equal and lower densities of *S. aureus* does not proceed beyond 8 h and shows deviations from pure *S. aureus*. At lower densities of *P. aeruginosa*, profiles which share more similiarites with *S. aureus* and shift in activity beyond 8 h is observed. This inhibition in the IMC is supported by the plate count data, and can be reasoned to be contributed by production of inhibitory substances.

In the case of mixed cultures with *E. coli*, although the profile of both species can be identified in the mixed culture at equal density, the profile of *E. coli* was particularly dominant when the density of *P. aeruginosa* in the mixed culture was between 10^5 to 10^3 CFU/mL. *P. aeruginosa* profile predominated in the mixed culture when the density of *E. coli* was between 10^4 to 10^3 CFU/mL. The plate count data supported that *P. aeruginosa* was inhibited in growth especially at 10^5 to 10^3 CFU/mL.

E. coli has been demonstrated to outgrow *P. aeruginosa* in biofilm cultures but outgrown in planktonic batch cultures (Culotti and Packman, 2014). Kuznetsova et al., (2013) also demonstrated using *E. coli* K12 TG1, a reference *P. aeruginosa* strain (ATCC 27853) and clinical *P. aeruginosa* strains (BALG and 9-3) that the CFU of *E. coli* in mixed culture with *P. aeruginosa* 27853 and 9-3 were significantly above that of the pure culture but that with *P. aeruginosa* BALG did not show any difference

after 12 h of incubation. For the CFU of *P. aeruginosa*, different effects were observed for the different strains in mixed culture; the CFU of *P. aeruginosa* BALG was significantly reduced. CFU of *P. aeruginosa* 27853 was significantly increased whilst that of *P. aeruginosa* 9-3 remained unchanged with respect to the pure cultures (Kuznetsova et al., 2013). In the present study, it was observed that the CFU of *E. coli* remains unchanged in the mixed culture with reference to the pure culture whilst that of *P. aeruginosa* decreased in all the mixed cultures but most appreciably when its initial inoculum density is lesser than *E. coli*. The inhibition of *P. aeruginosa* may be due to nutrient competition, reduced virulence in mixed cultures with *E. coli* and or the production of metabolites by *E. coli*. Further studies need to be done to substantiate and understand the underlying mechanism of inhibition of *P. aeruginosa*.

4. Conclusions

In conclusion, the results presented in this paper corroborates previous studies and demonstrate that IMC could be a valuable tool for studying behaviour of mixed cultures and could potentially be used for studying clinically relevant interaction as it opens up a lot of possibilities. The results from the IMC underpin that interaction occurs in polymicrobial communities and suggest a characterization trend towards mixed microbial assays.

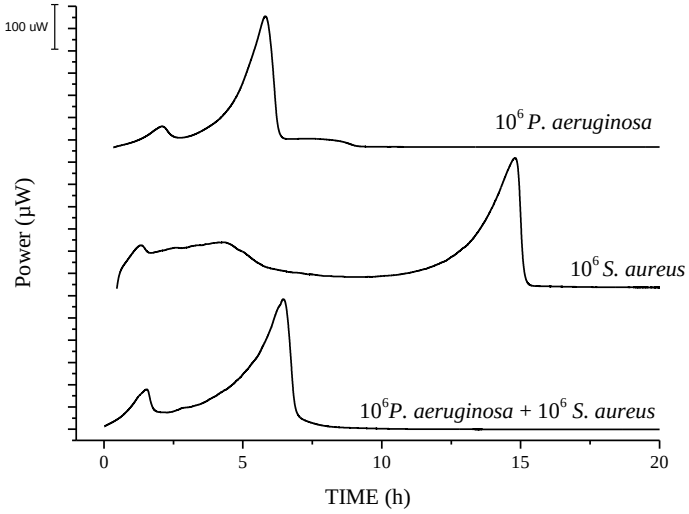

Fig. 1. Comparison of the power-time growth curves of pure cultures of *P. aeruginosa, S. aureus* and a mixed culture in NB at same inoculum density (CFU/mL). Growth curve of mixed culture share similarities with *P. aeruginosa*

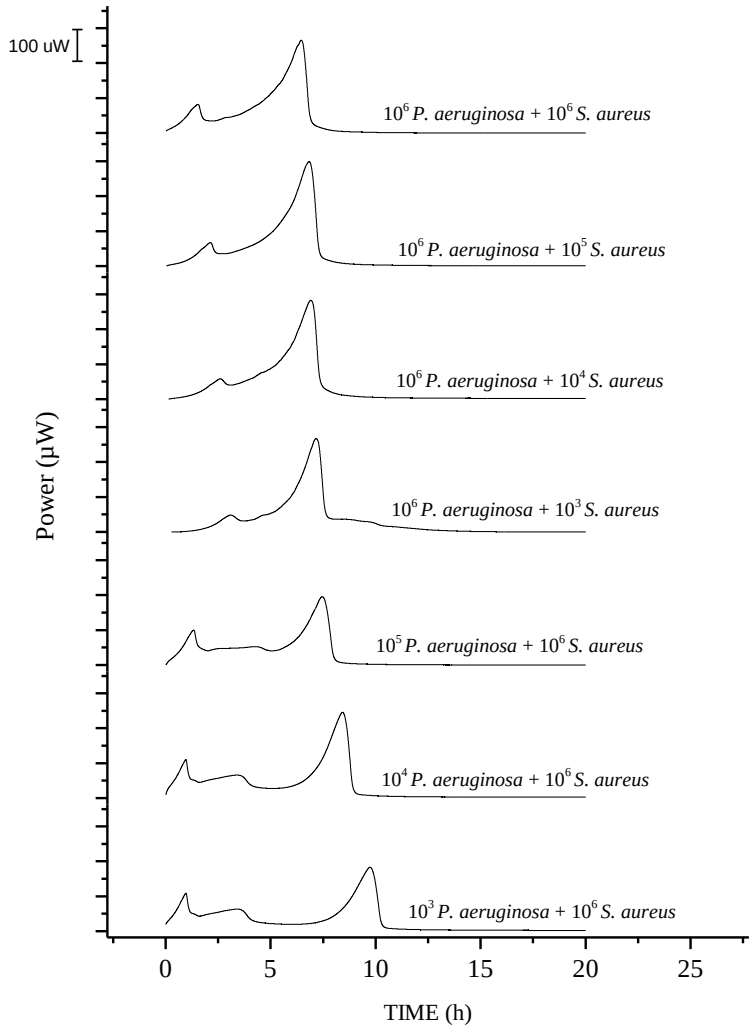

Fig. 2. Comparison of power-time curves of mixed cultures of *P. aeruginosa* and *S. aureus* when the density of *P. aeruginosa* was kept constant at 10^6 CFU/mL and that of *S. aureus* decreased from 10^6 to 10^3 CFU/mL and vice versa. The growth curves show that mixed cultures reverts to the pure species as density of one of the species is decreased over the other

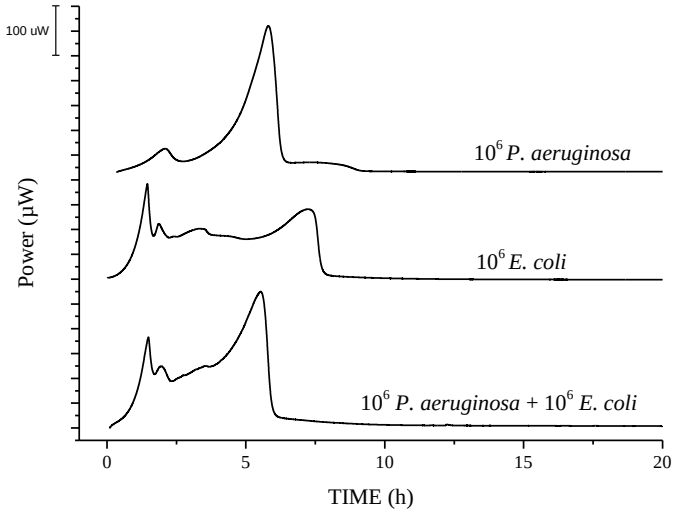

Fig. 3. Comparison of the power-time curves of *P. aeruginosa, E.coli* and a mixed culture in NB at same inoculum density (CFU/mL)

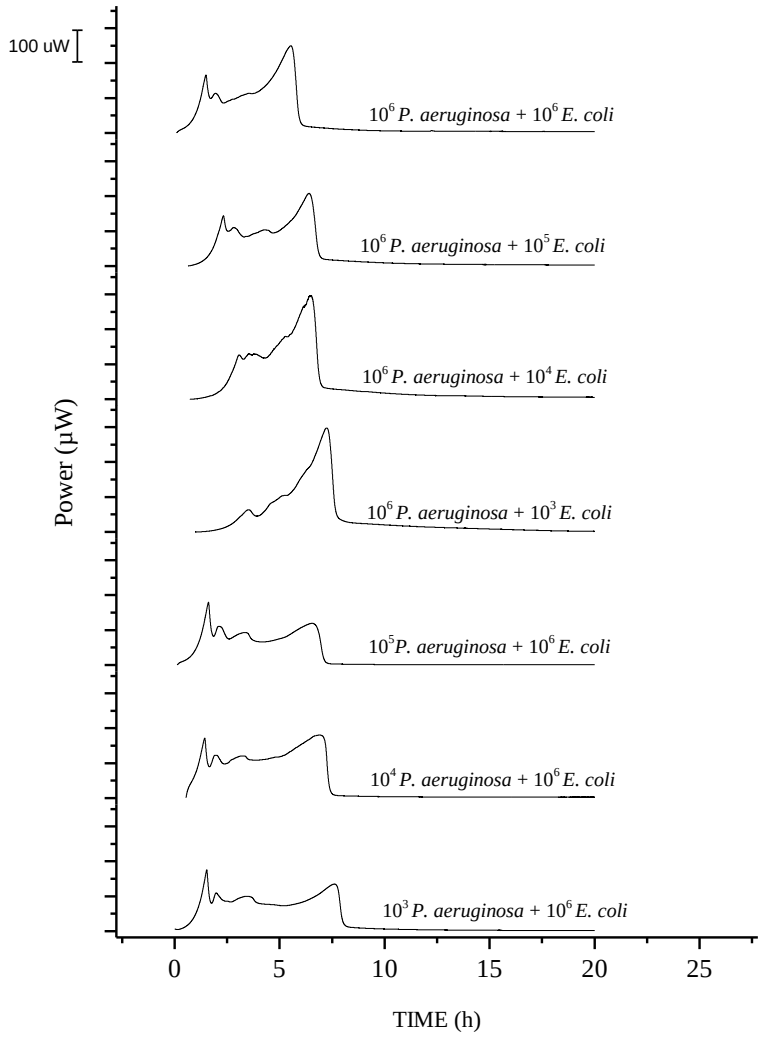

Fig. 4. Comparison of power-time curves of mixed cultures of *P. aeruginosa* and *E.coli* when the density of *P. aeruginosa* was kept constant at 10^6 CFU/mL and that of *E. coli* decreased from 10^6 to 10^3 CFU/mL and vice versa. The growth curves show that mixed cultures reverts to the pure species as density of one of the species is decreased over the other

Table 1. Total heat output (Q_t), first peak (P_1), time of registration of first peak (t_1), final metabolic/maximum peak (P_{fmax}), time of registration of final metabolic/maximum peak (t_{fmax}) of *P. aerugionsa*, *S. aureus* and their mixed cultures

Microorganism (CFU/mL)	Q_t (J)	P_1 (μW)	t_1 (h)	P_{fmax} (μW)	t_{fmax} (h)
10^6 *P. aeruginosa*	1.77 ± 0.12	46.14 ± 1.35	2.10 ± 0.01	287.06 ± 29.80	5.85 ± 0.05
10^6 *S. aureus*	3.91 ± 0.83	124.88 ± 23.73	1.02 ± 0.29	263.62 ± 68.34	14.94 ± 0.42
10^6 *P. aeruginosa* + 10^6 *S. aureus*	2.46 ± 0.08	68.42 ± 29.61	1.61 ± 0.10	282.58 ± 13.57	6.20 ± 0.38
10^6 *P. aeruginosa* + 10^5 *S. aureus*	2.62 ± 0.13	76.99 ± 5.29	2.16 ± 0.08	339.08 ± 14.92	6.42 ± 0.55
10^6 *P. aeruginosa* + 10^4 *S. aureus*	2.55 ± 0.22	69.97 ± 9.01	2.51 ± 0.19	301.05 ± 15.13	6.52 ± 0.55
10^6 *P. aeruginosa* + 10^3 *S. aureus*	2.28 ± 0.12	64.36 ± 16.70	2.79 ± 0.45	317.052 ± 33.30	6.68 ± 0.73
10^5 *P. aeruginosa* + 10^6 *S. aureus*	2.32 ± 0.17	112.0 ± 3.25	1.35 ± 0.04	223.22 ± 11.68	8.25 ± 1.14
10^4 *P. aeruginosa* + 10^6 *S. aureus*	2.68 ± 0.14	120.05 ± 1.59	0.97 ± 0.01	270.08 ± 0.152	8.42 ± 0.01
10^3 *P. aeruginosa* + 10^6 *S. aureus*	2.38 ± 0.08	114.04 ± 6.79	0.10 ± 0.02	219.98 ± 26.27	9.79 ± 0.08

Table 2. pH determination and viable count after IMC experiments of *P. aeruginosa*, *S. aureus* and their mixed cultures

Microorganism (CFU/mL)	pH	Plate count (log CFU/mL)	
10^6 *P. aeruginosa*	6.8 ± 0.2	7.8 ± 0.1	
10^6 *S. aureus*	6.3 ± 0.2	8.1 ± 0.1	
		P. aeruginosa	*S. aureus*
10^6 *P. aeruginosa* + 10^6 *S. aureus*	6.3 ± 0.3	7.4 ± 0.2	5.7 ± 0.3
10^6 *P. aeruginosa* + 10^5 *S. aureus*	6.4 ± 0.1	7.4 ± 0.3	6.5 ± 0.1
10^6 *P. aeruginosa* + 10^4 *S. aureus*	6.4 ± 0.2	7.2 ± 0.4	6.0 ± 0.5
10^6 *P. aeruginosa* + 10^3 *S. aureus*	6.5 ± 0.2	7.6 ± 0.2	3.1 ± 0.2
10^5 *P. aeruginosa* + 10^6 *S. aureus*	6.4 ± 0.1	7.4 ± 0.2	7.1 ± 0.2
10^4 *P. aeruginosa* + 10^6 *S. aureus*	6.4 ± 0.2	7.4 ± 0.3	7.4 ± 0.2
10^3 *P. aeruginosa* + 10^6 *S. aureus*	6.3 ± 0.1	7.1 ± 0.2	7.2 ± 0.4

Table 3. Total heat output (Q_t), first peak (P_1), time of registration of first peak (t_1), final metabolic/maximum peak (P_{fmax}), time of registration of final metabolic/maximum peak (t_{fmax}) of *P. aerugionsa*, *E. coli* and their mixed cultures

Microorganism (CFU/mL)	Q_t (J)	P_1 (μW)	t_1 (h)	P_{fmax} (μW)	t_{fmax} (h)
10^6 *P. aeruginosa*	1.77 ± 0.20	46.14 ± 1.35	2.10 ± 0.01	287.06 ± 29.80	5.85 ± 0.05
10^6 *E. coli*	3.12 ± 0.52	200.51 ± 15.69	1.50 ± 0.05	131.54 ± 20.52	7.82 ± 0.50
10^6 *P. aeruginosa* + 10^6 *E. coli*	2.71 ± 0.10	179.19 ± 4.61	1.57 ± 0.12	284.56 ± 14.02	5.67 ± 0.21
10^6 *P. aeruginosa* + 10^5 *E. coli*	2.58 ± 0.25	141.29 ± 18.88	2.24 ± 0.08	269.08 ± 58.66	6.02 ± 0.59
10^6 *P. aeruginosa* + 10^4 *E. coli*	3.25 ± 0.45	111.49 ± 37.49	2.77 ± 0.38	316.81 ± 11.59	6.23 ± 0.37
10^6 *P. aeruginosa* + 10^3 *E. coli*	2.75 ± 0.38	73.57 ± 6.12	3.07 ± 0.59	311.48 ± 21.95	6.53 ± 0.96
10^5 *P. aeruginosa* + 10^6 *E. coli*	2.24 ± 0.03	154.77 ± 58.14	1.47 ± 0.18	181.19 ± 71.12	6.82 ± 0.33
10^4 *P. aeruginosa* + 10^6 *E. coli*	3.09 ± 0.21	193.68 ± 6.62	1.46 ± 0.06	145.85 ± 73.75	7.04 ± 0.08
10^3 *P. aeruginosa* + 10^6 *E. coli*	2.71 ± 0.16	191.10 ± 3.40	1.51 ± 0.01	148.22 ± 0.04	7.62 ± 0.01

Table 4. pH determination and viable count after IMC experiments of *P. aeruginosa*, *E. coli* and their mixed cultures

Microorganism (CFU/mL)	pH	Plate count after experiments (log CFU/mL)	
10^6 *P. aeruginosa*	6.8 ± 0.2	7.8 ± 0.1	
10^6 *E. coli*	5.2 ± 0.3	8.2 ± 0.2	
		P. aeruginosa	*E. coli*
10^6 *P. aeruginosa* + 10^6 *E. coli*	5.4 ± 0.1	6.9 ± 0.5	8.1 ± 0.1
10^6 *P. aeruginosa* + 10^5 *E. coli*	5.5 ± 0.2	6.8 ± 0.7	8.2 ± 0.2
10^6 *P. aeruginosa* + 10^4 *E. coli*	5.8 ± 0.3	6.7 ± 0.1	8.2 ± 0.2
10^6 *P. aeruginosa* + 10^3 *E. coli*	5.9 ± 0.2	7.4 ± 0.2	8.2 ± 0.1
10^5 *P. aeruginosa* + 10^6 *E. coli*	5.4 ± 0.1	5.0 ± 0.3	8.3 ± 0.2
10^4 *P. aeruginosa* + 10^6 *E. coli*	5.5 ± 0.2	4.7 ± 0.1	8.2 ± 0.2
10^3 *P. aeruginosa* + 10^6 *E. coli*	5.6 ± 0.2	4.5 ± 0.3	8.2 ± 0.1

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