Appendices

Appendix A: Statistical analysis of the effect of temperature on the susceptibility of *P.aruginosa*

Table A.1: Statistical analysis of inhibition zone size (mm) of colistin using the agar well diffusion method

Descriptive Statistics

Colistin (µg/ml)	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
25°C						
16	2	13	14	13.50	.500	.707
8	2	11.00	12.00	11.5000	.50000	.70711
4	2	.00	.00	.0000	.00000	.00000
2	2	.00	.00	.0000	.00000	.00000
1	2	.00	.00	.0000	.00000	.00000
0.5	2	.00	.00	.0000	.00000	.00000
30°C	0					
16	4	17.00	18.00	17.5000	.28868	.57735
8	4	14.00	15.00	14.2500	.25000	.50000
4	2	11.00	11.00	11.0000	.00000	.00000
2	2	.00	.00	.0000	.00000	.00000
1	2	.00	.00	.0000	.00000	.00000
37°C	0					
16	4	18.00	19.00	18.7500	.25000	.50000
8	3	15.00	15.00	15.0000	.00000	.00000
4	2	12.00	12.00	12.0000	.00000	.00000
2	2	.00	.00	.0000	.00000	.00000
1	2	.00	.00	.0000	.00000	.00000
42°C	0					
16	3	21.00	21.00	21.0000	.00000	.00000
8	3	18.00	18.00	18.0000	.00000	.00000
4	3	14.00	16.00	15.0000	.57735	1.00000
2	3	12.00	12.00	12.0000	.00000	.00000
1	2	.00	.00	.0000	.00000	.00000
0.5	2	.00	.00	.0000	.00000	.00000
Valid N (listwise)	0					

Table A.2: Statistical analysis of inhibition zone size (mm) of amikacin using the agar well diffusion method

Descriptive Statistics

Amikacin (µg/ml)	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
25°C						
20	4	14	15	14.75	.250	.500
10	2	12.00	13.00	12.5000	.50000	.70711
5	4	.00	.00	.0000	.00000	.00000
2.5	4	.00	.00	.0000	.00000	.00000
1.25	4	.00	.00	.0000	.00000	.00000
0.6	4	.00	.00	.0000	.00000	.00000
3°0C						
20	3	16.00	16.00	16.0000	.00000	.00000
10	3	12.00	13.00	12.6667	.33333	.57735
5	3	.00	.00	.0000	.00000	.00000
2.5	3	.00	.00	.0000	.00000	.00000
37°C						
20	3	19.00	19.00	19.0000	.00000	.00000
10	3	16.00	17.00	16.3333	.33333	.57735
5	3	.00	.00	.0000	.00000	.00000
2.5	3	.00	.00	.0000	.00000	.00000
1.25	3	.00	.00	.0000	.00000	.00000
0.6	3	.00	.00	.0000	.00000	.00000
42°C						
20	3	24.00	25.00	24.6667	.33333	.57735
10	3	18.00	20.00	19.3333	.66667	1.15470
5	3	15.00	15.00	15.0000	.00000	.00000
2.5	3	.00	.00	.0000	.00000	.00000
1.25	3	.00	.00	.0000	.00000	.00000
0.6	3	.00	.00	.0000	.00000	.00000
Valid N (listwise)						

Table A.3: Statistical analysis of inhibition zone size (mm) of tobramycin using the agar well diffusion method

Descriptive Statistics

Tobramycin (µg/ml)	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
25°C						
16	2	16	17	16.50	.500	.707
8	2	14.00	14.00	14.0000	.00000	.00000
4	2	.00	.00	.0000	.00000	.00000
2	2	.00	.00	.0000	.00000	.00000
1	2	.00	.00	.0000	.00000	.00000
0.5	2	.00	.00	.0000	.00000	.00000
30°C						
16	4	20.00	21.00	20.5000	.28868	.57735
8	3	16.00	17.00	16.6667	.33333	.57735
4	3	12.00	13.50	12.8333	.44096	.76376
2	2	11.00	11.00	11.0000	.00000	.00000
1	2	.00	.00	.0000	.00000	.00000
37°C						
16	4	22.00	22.00	22.0000	.00000	.00000
8	3	18.00	20.00	19.3333	.66667	1.15470
4	4	16.00	16.00	16.0000	.00000	.00000
2	2	14.00	14.00	14.0000	.00000	.00000
1	2	.00	.00	.0000	.00000	.00000
42°C						
16	2	25.00	25.00	25.0000	.00000	.00000
8	2	21.00	21.00	21.0000	.00000	.00000
4	2	15.00	16.00	15.5000	.50000	.70711
2	2	13.00	14.00	13.5000	.50000	.70711
1	2	.00	.00	.0000	.00000	.00000
0.5	2	.00	.00	.0000	.00000	.00000
Valid N (listwise)	0					

Table A.4. The correlation between the MIC and temperatures using different methods. (A) Amikacin (B) tobramycin, and (C) colistin.

	OxoPlate	E-test	Agar Well Diffusion
A.	**Correlations** T: Pearson Correlation 1, -.902; Sig. (2-tailed) .098; N 4, 4 MIC: Pearson Correlation -.902, 1; Sig. (2-tailed) .098; N 4, 4	**Correlations** T: Pearson Correlation 1, -.972*; Sig. (2-tailed) .028; N 4, 4 MIC: Pearson Correlation -.972*, 1; Sig. (2-tailed) .028; N 4, 4 *. Correlation is significant at the 0.05 level (2-tailed).	**Correlations** T: Pearson Correlation 1, -.755; Sig. (2-tailed) .245; N 4, 4 MIC: Pearson Correlation -.755, 1; Sig. (2-tailed) .245; N 4, 4
B.	**Correlations** MIC: Pearson Correlation 1, -.911; Sig. (2-tailed) .089; N 4, 4 T: Pearson Correlation -.911, 1; Sig. (2-tailed) .089; N 4, 4	**Correlations** T: Pearson Correlation 1, -.891; Sig. (2-tailed) .109; N 4, 4 MIC: Pearson Correlation -.891, 1; Sig. (2-tailed) .109; N 4, 4	**Correlations** T: Pearson Correlation 1, -.755; Sig. (2-tailed) .245; N 4, 4 MIC: Pearson Correlation -.755, 1; Sig. (2-tailed) .245; N 4, 4
C.	**Correlations** T: Pearson Correlation 1, -.923; Sig. (2-tailed) .077; N 4, 4 MIC: Pearson Correlation -.923, 1; Sig. (2-tailed) .077; N 4, 4	**Correlations** T: Pearson Correlation 1, -.976*; Sig. (2-tailed) .024; N 4, 4 MIC: Pearson Correlation -.976*, 1; Sig. (2-tailed) .024; N 4, 4 *. Correlation is significant at the 0.05 level (2-tailed).	**Correlations** T: Pearson Correlation 1, -.900; Sig. (2-tailed) .100; N 4, 4 MIC: Pearson Correlation -.900, 1; Sig. (2-tailed) .100; N 4, 4

Appendix B: Susceptibility of *P.aeruginosa* in the presence of divalent cation

1. Effect of divalent cation individually on the susceptibility of *P. aeruginosa* to antibiotics

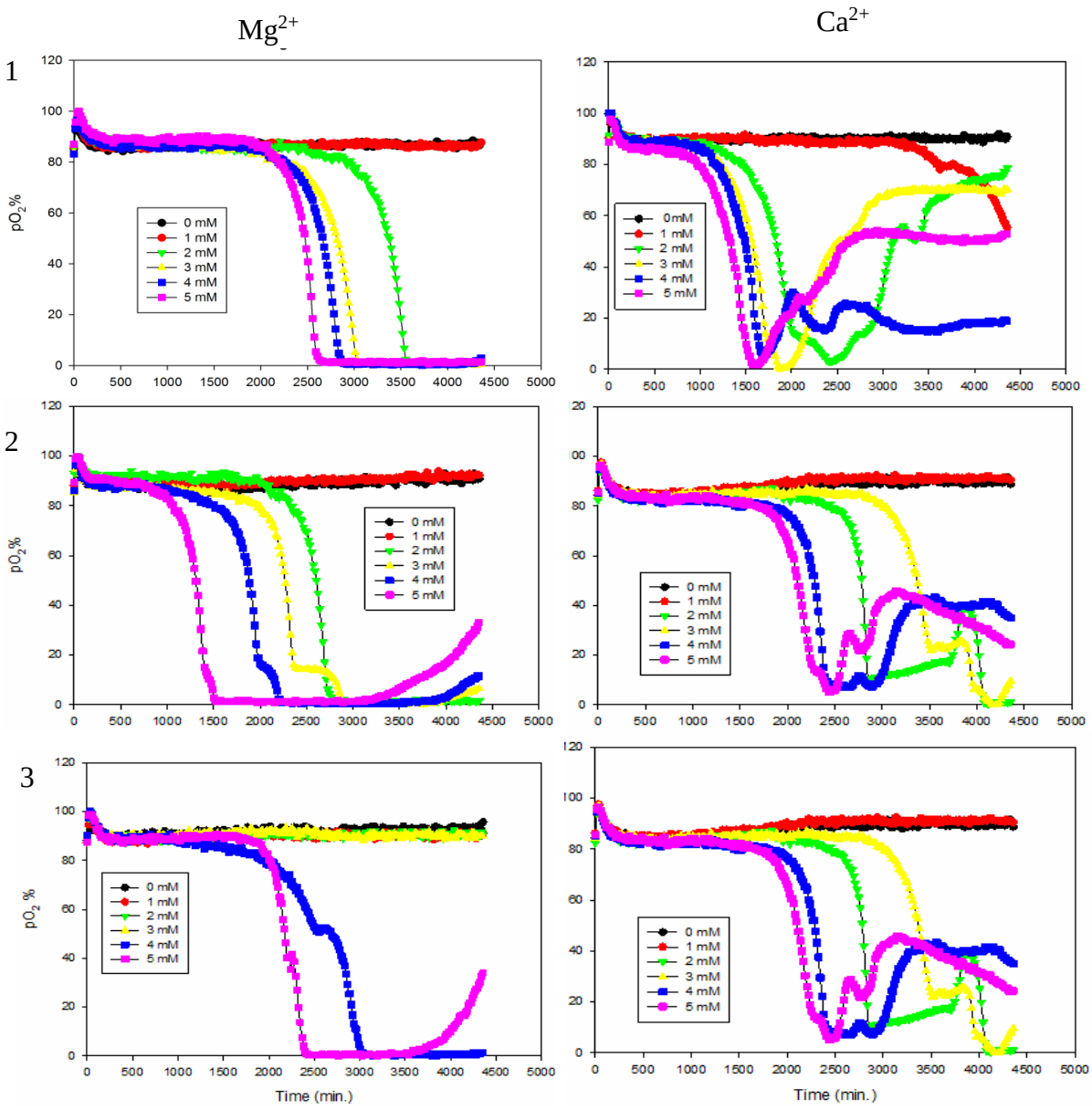
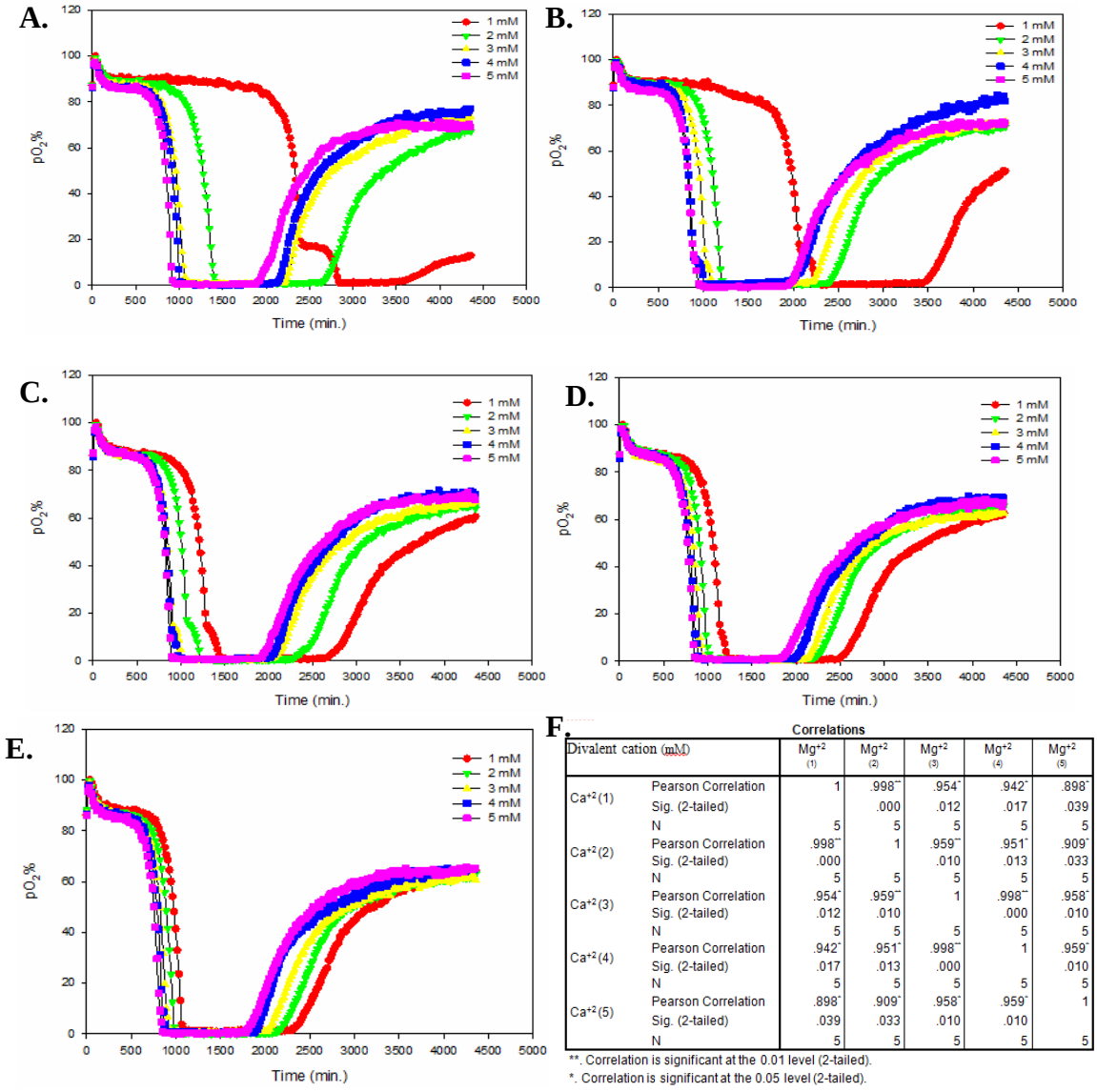


Figure B.1: The effect of various concentrations of Mg^{2+} and Ca^{2+} (1-5mM) on PA01 treated with colistin at 2µg/ ml (1), amikacin at 2.5 µg/ ml (2) and tobramycin at 0.5 µg/ ml (3).

2. Effect of Mg^{2+} in combination with Ca^{2+} on the *in-vitro* susceptibility of *P. aeruginosa* to antibiotics



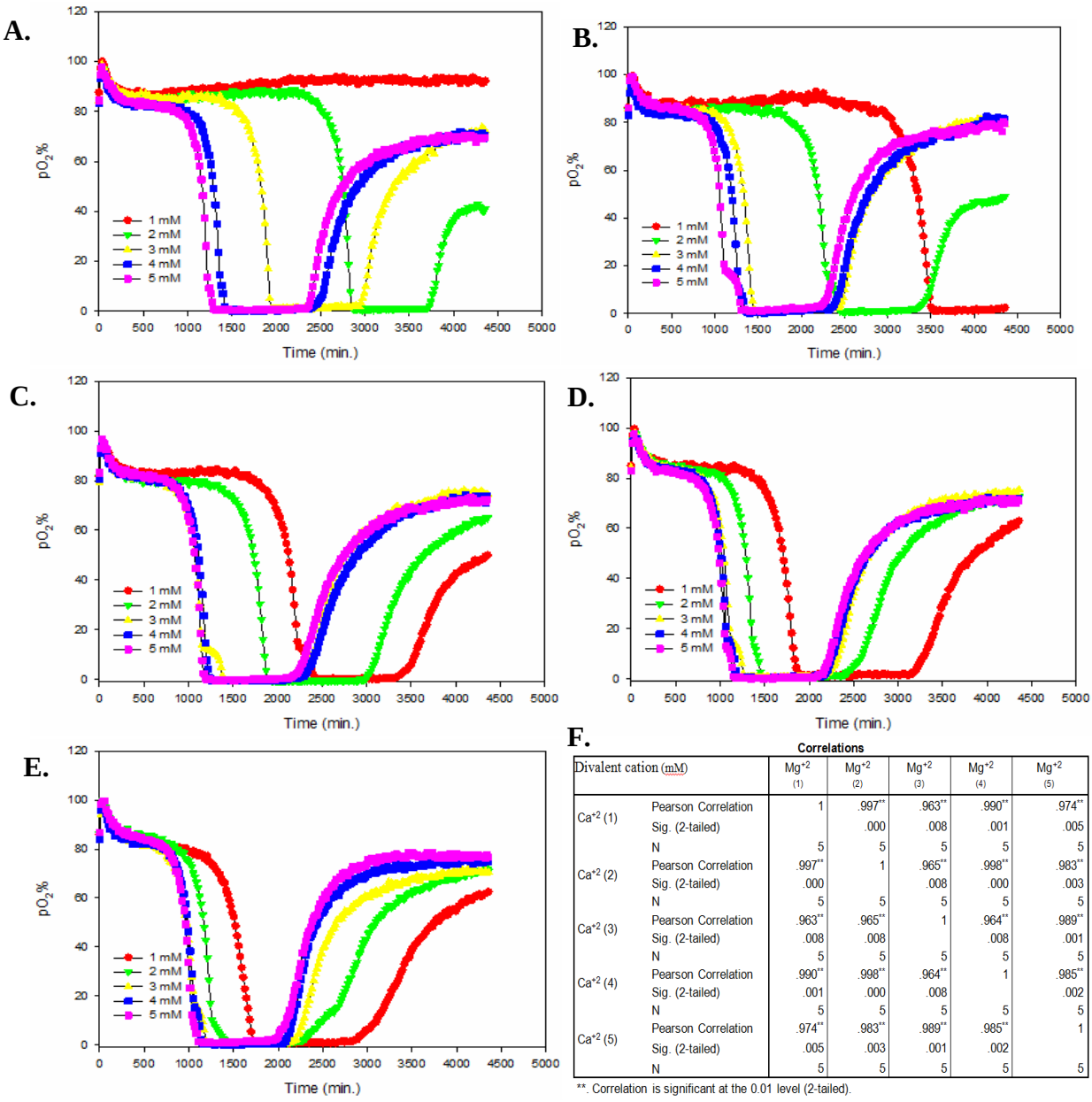
F.

Correlations

Divalent cation (mM)		Mg^{+2} (1)	Mg^{+2} (2)	Mg^{+2} (3)	Mg^{+2} (4)	Mg^{+2} (5)
Ca^{+2}(1)	Pearson Correlation	1	.998**	.954*	.942*	.898*
	Sig. (2-tailed)		.000	.012	.017	.039
	N	5	5	5	5	5
Ca^{+2}(2)	Pearson Correlation	.998**	1	.959**	.951*	.909*
	Sig. (2-tailed)	.000		.010	.013	.033
	N	5	5	5	5	5
Ca^{+2}(3)	Pearson Correlation	.954*	.959**	1	.998**	.958*
	Sig. (2-tailed)	.012	.010		.000	.010
	N	5	5	5	5	5
Ca^{+2}(4)	Pearson Correlation	.942*	.951*	.998**	1	.959*
	Sig. (2-tailed)	.017	.013	.000		.010
	N	5	5	5	5	5
Ca^{+2}(5)	Pearson Correlation	.898*	.909*	.958*	.959*	1
	Sig. (2-tailed)	.039	.033	.010	.010	
	N	5	5	5	5	5

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Figure B.2: Effect of various concentration of Ca^{2+} combined with a constant concentration of Mg^{2+} A. 1mM , **B.** 2mM, **C.** 3mM ,**D**. 4mM and **E.** 5mM **on growth inhibition of PA01.** The bacterial cultures incubated at 30C° for 72h with 2.5 ug/ml of amikacin. The values shown are the means of two replicates. F represents the correlation between "time to detect" and ions.



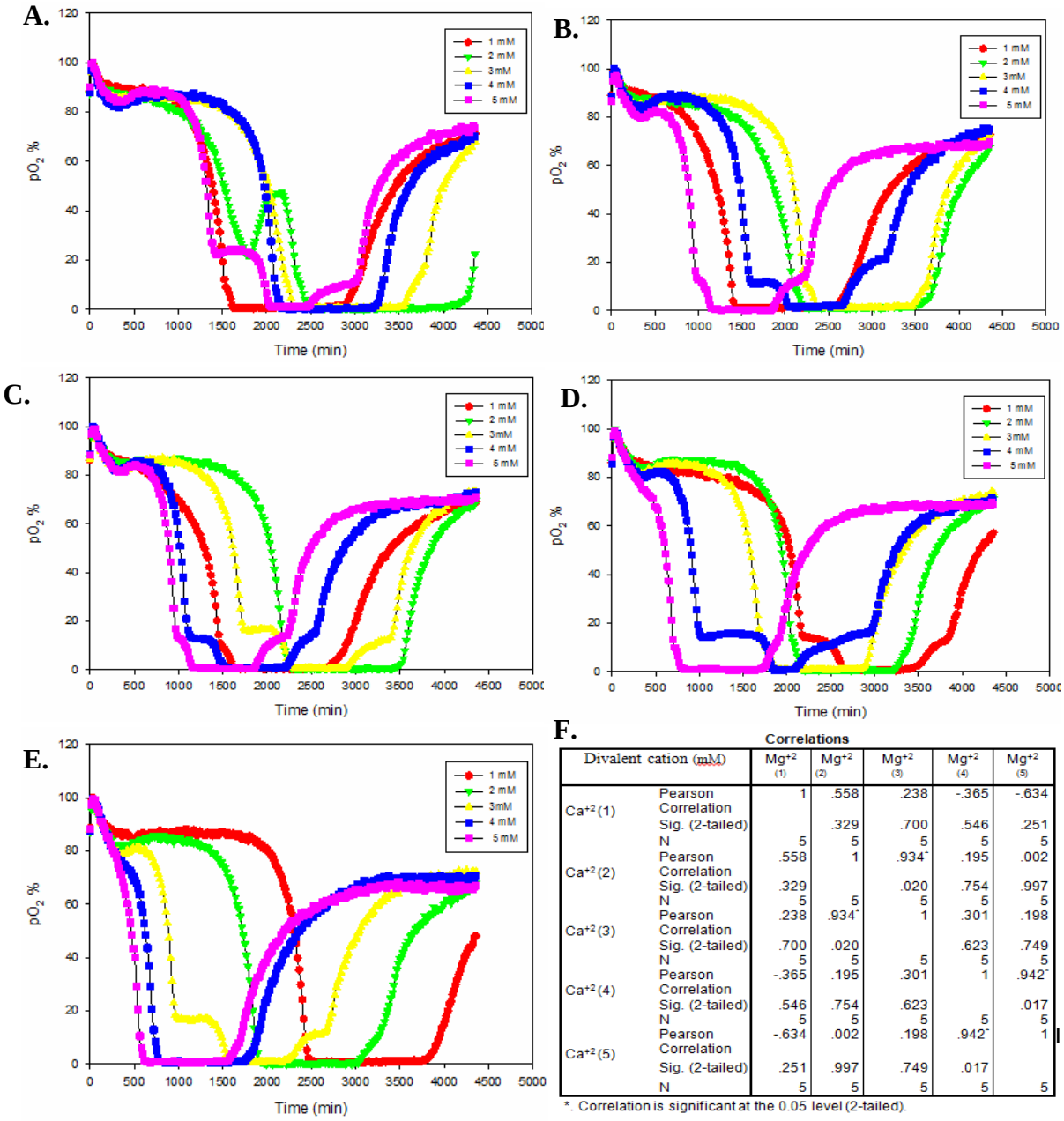
F.

Correlations

Divalent cation (mM)		Mg^{+2} (1)	Mg^{+2} (2)	Mg^{+2} (3)	Mg^{+2} (4)	Mg^{+2} (5)
Ca^{+2} (1)	Pearson Correlation	1	.997**	.963**	.990**	.974**
	Sig. (2-tailed)		.000	.008	.001	.005
	N	5	5	5	5	5
Ca^{+2} (2)	Pearson Correlation	.997**	1	.965**	.998**	.983**
	Sig. (2-tailed)	.000		.008	.000	.003
	N	5	5	5	5	5
Ca^{+2} (3)	Pearson Correlation	.963**	.965**	1	.964**	.989**
	Sig. (2-tailed)	.008	.008		.008	.001
	N	5	5	5	5	5
Ca^{+2} (4)	Pearson Correlation	.990**	.998**	.964**	1	.985**
	Sig. (2-tailed)	.001	.000	.008		.002
	N	5	5	5	5	5
Ca^{+2} (5)	Pearson Correlation	.974**	.983**	.989**	.985**	1
	Sig. (2-tailed)	.005	.003	.001	.002	
	N	5	5	5	5	5

**. Correlation is significant at the 0.01 level (2-tailed).

Figure B.3: Effect of various concentration of Ca^{2+} combined with a constant concentration of Mg^{2+} A. 1mM , **B.** 2mM, **C.** 3mM ,**D**. 4mM and **E.** 5mM **on growth inhibition of PA01.** The bacterial cultures incubated at 30C° for 72h with 0.5ug/ml of tobramycin. The values shown are the means of two replicates. F represents the correlation between " time to detect" and ions.



F.

Correlations

Divalent cation (mM)		Mg+2 (1)	Mg+2 (2)	Mg+2 (3)	Mg+2 (4)	Mg+2 (5)
Ca+2 (1)	Pearson Correlation	1	.558	.238	-.365	-.634
	Sig. (2-tailed)		.329	.700	.546	.251
	N	5	5	5	5	5
Ca+2 (2)	Pearson Correlation	.558	1	.934*	.195	.002
	Sig. (2-tailed)	.329		.020	.754	.997
	N	5	5	5	5	5
Ca+2 (3)	Pearson Correlation	.238	.934*	1	.301	.198
	Sig. (2-tailed)	.700	.020		.623	.749
	N	5	5	5	5	5
Ca+2 (4)	Pearson Correlation	-.365	.195	.301	1	.942*
	Sig. (2-tailed)	.546	.754	.623		.017
	N	5	5	5	5	5
Ca+2 (5)	Pearson Correlation	-.634	.002	.198	.942*	1
	Sig. (2-tailed)	.251	.997	.749	.017	
	N	5	5	5	5	5

*. Correlation is significant at the 0.05 level (2-tailed).

Figure B.4: Effect of various concentration of Ca^{2+} combined with a constant concentration of Mg^{2+} A. 1mM , **B.** 2mM, **C.** 3mM ,**D**. 4mM and **E.** 5mM **on growth inhibition of PA01.** The bacterial cultures incubated at 30C° for 72h with 2ug/ml of colistin. The values shown are the means of two replicates. F represents the correlation between "time to detect" and ions.

Appendix C. Growth rate of *P.aeruginosa* in the presence of divalent cation

1. Control cultures

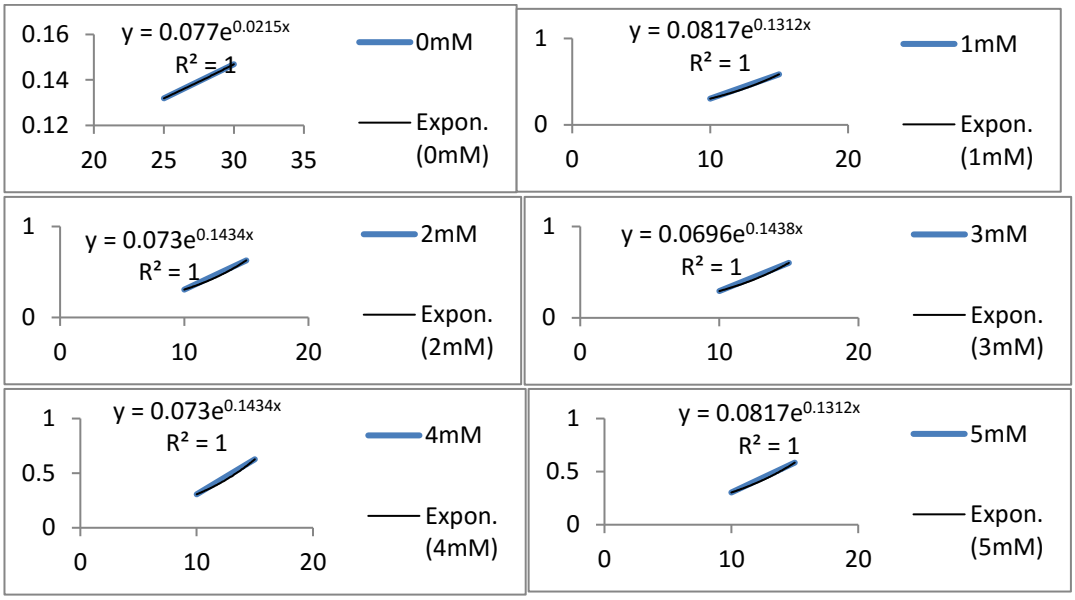


Figure C.1: The growth rate of *P.aeruginosa* in the presence of Mg^{2+}

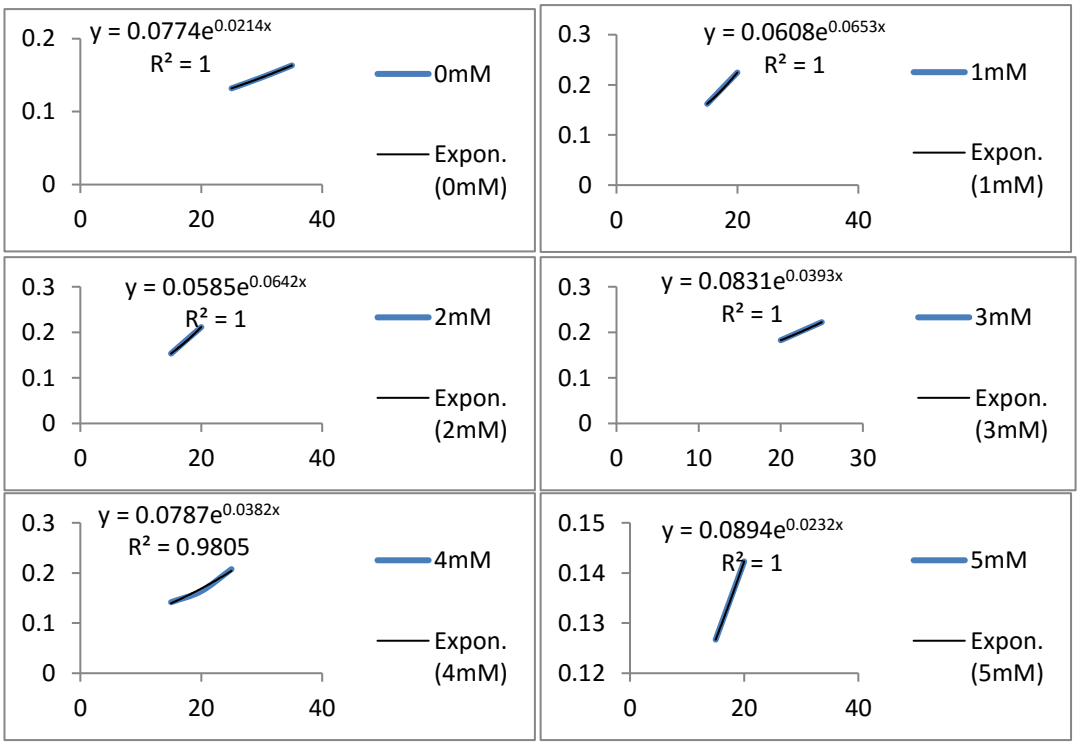


Figure C.2: The growth rate of *P.aeruginosa* in the presence of Ca^{2+}

2. In the presence of antibiotics

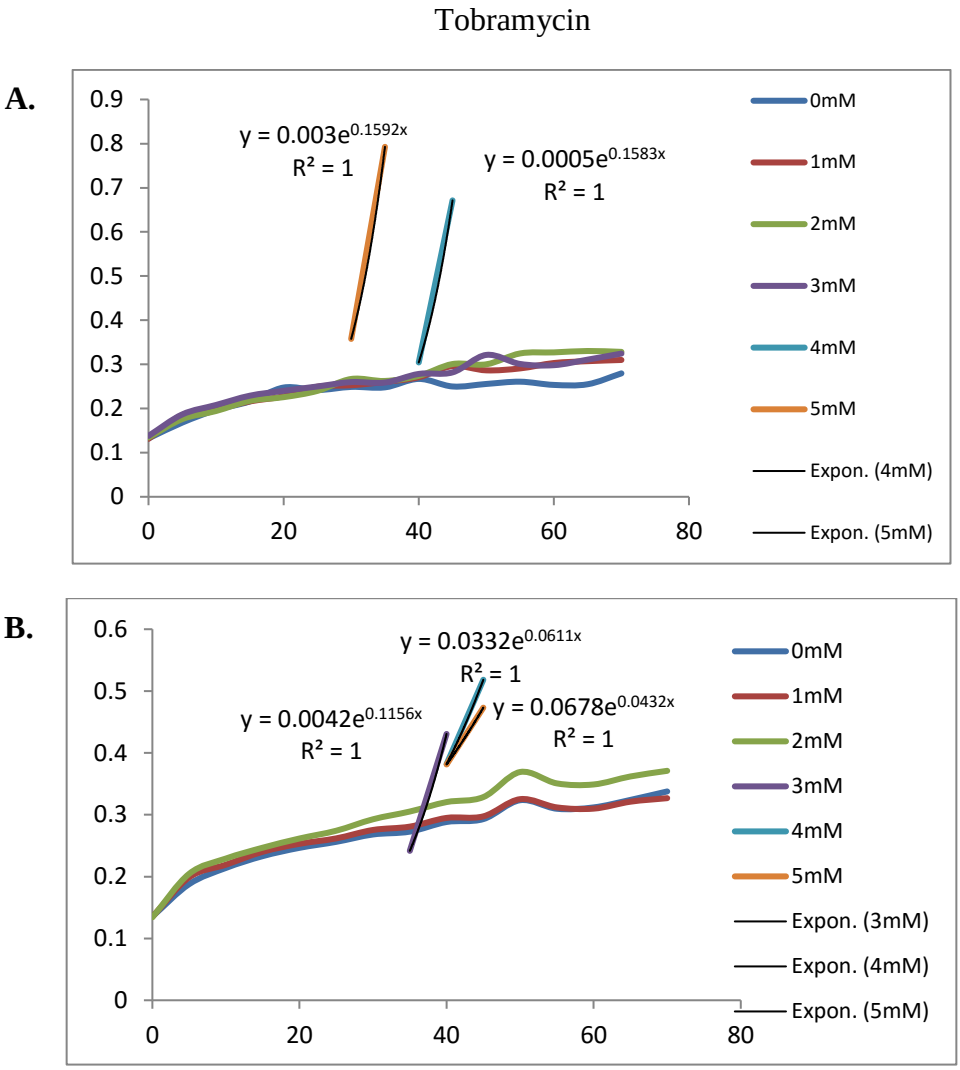
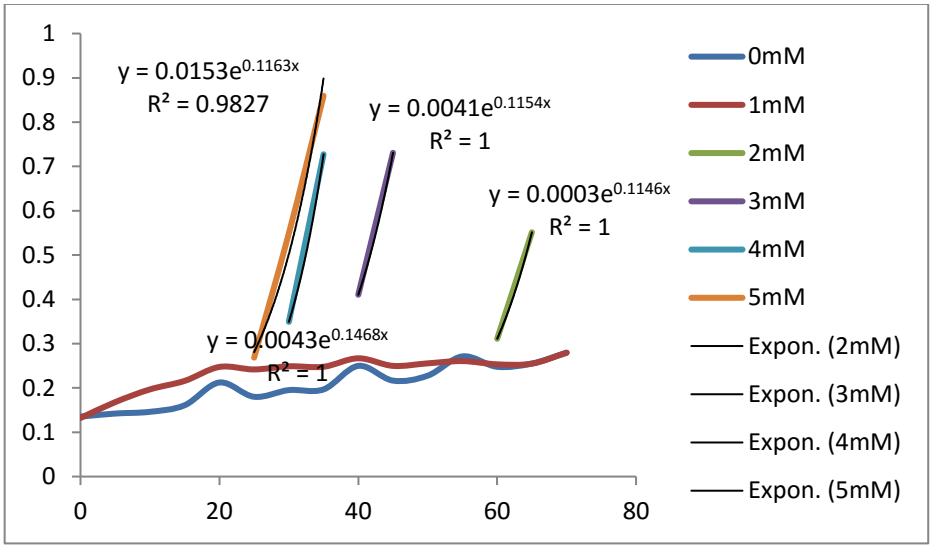


Figure C.3: The growth rate of *P.aeruginosa* in the presence of Mg^{2+} (A) or Ca^{2+} (B)

Amikacin

A.



B.

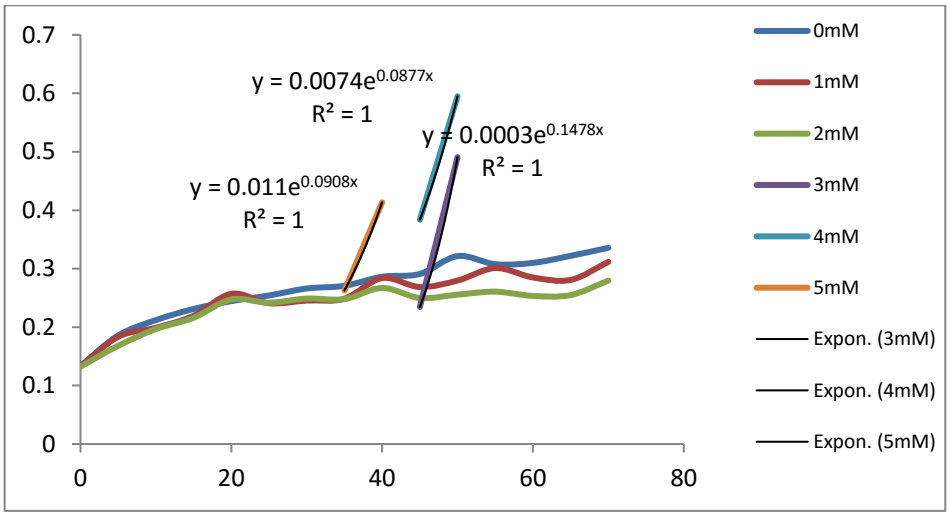


Figure C.4: The growth rate of *P.aeruginosa* in the presence of Mg^{2+} (A) or Ca^{2+} (B)

Colistin

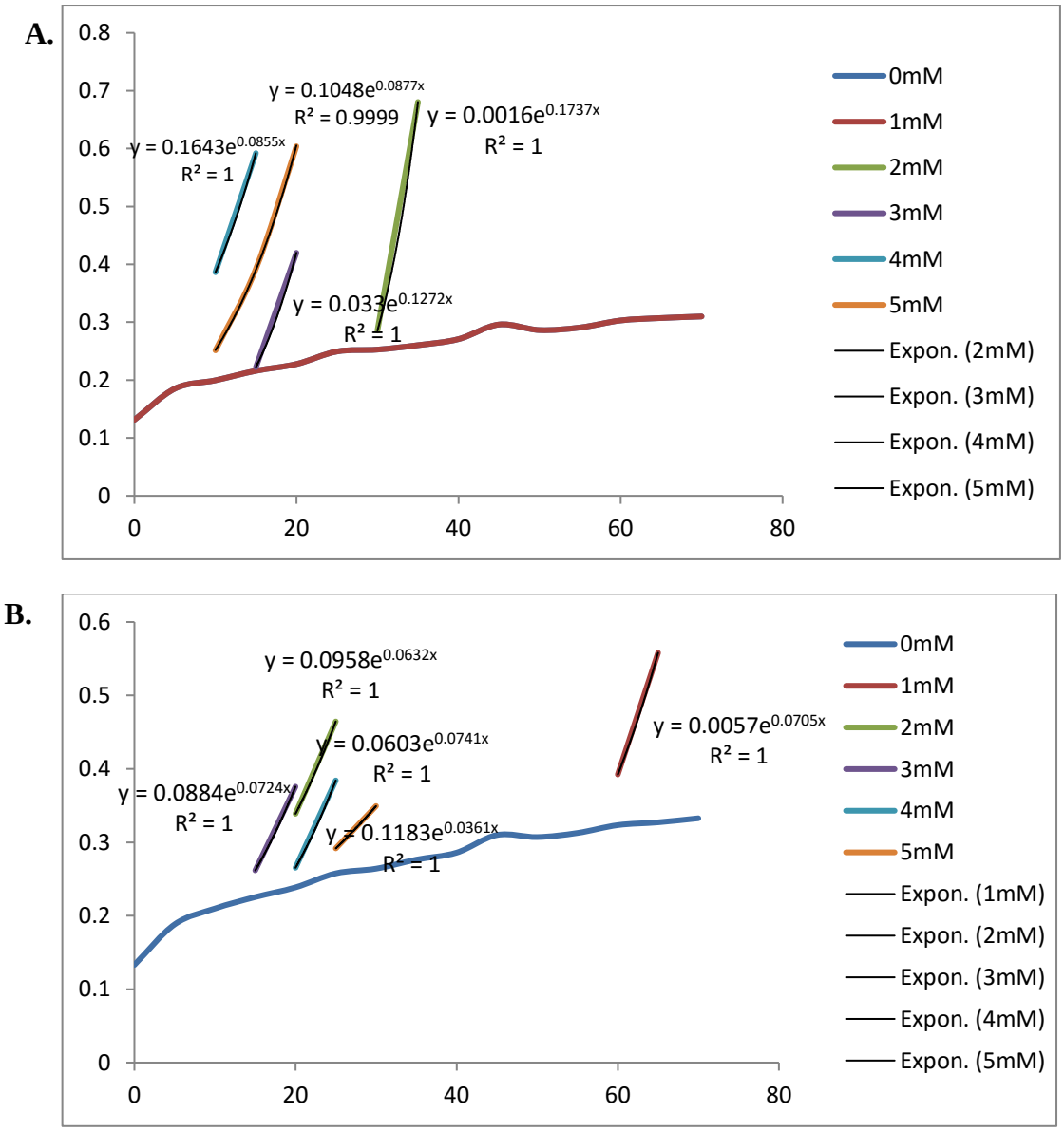


Figure C.5: The growth rate of *P.aeruginosa* in the presence of Mg^{2+} (A) or Ca^{2+} (B)

Appendix D: Effect of inoculum size on the susceptibility of *P.aeruginosa*

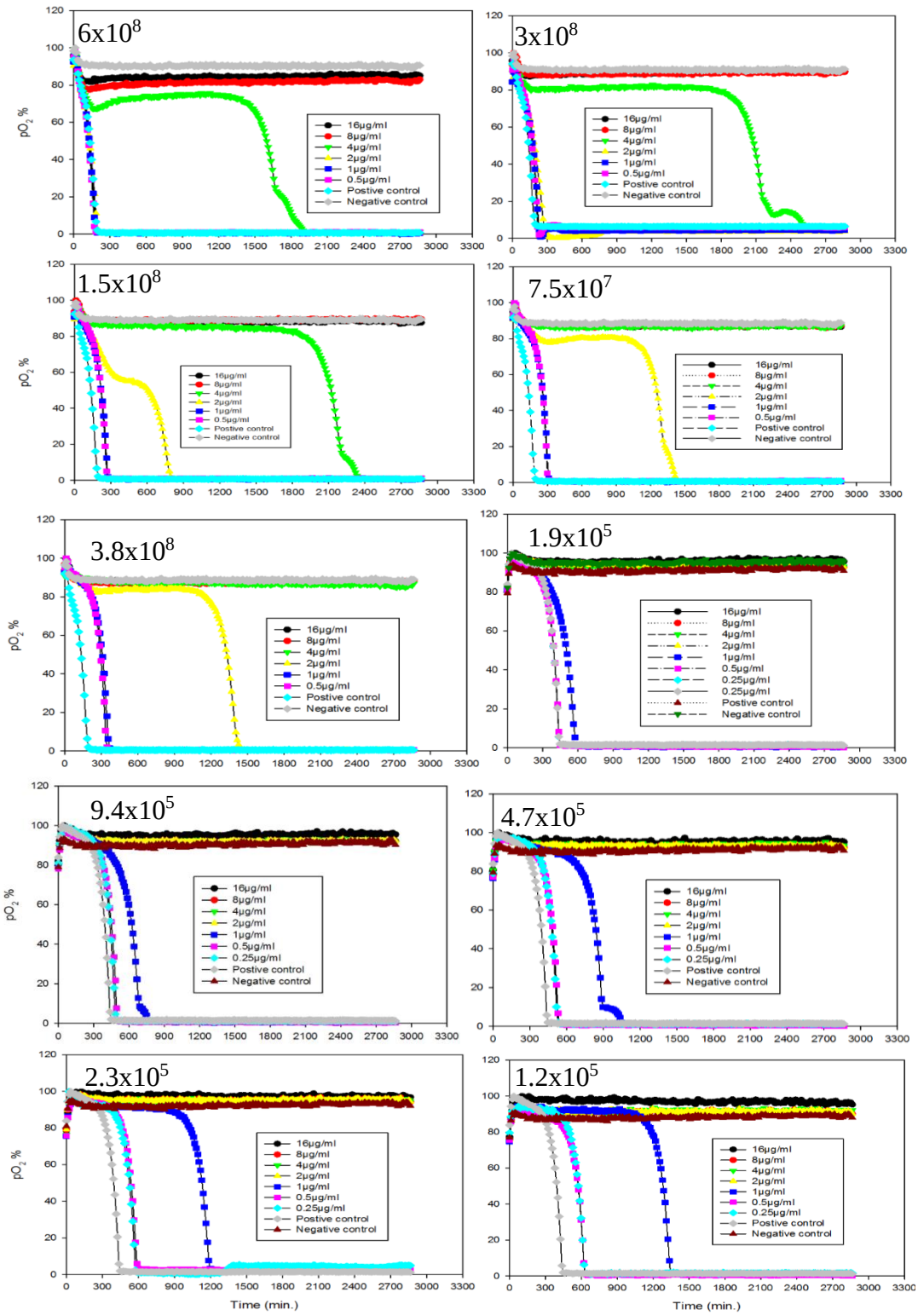


Figure D.1: Inoculum effect on MIC and MBC of tobramycin. Planktonic cells of *P.aeruginosa* were treated with tobramycin at different concentrations from 16-0.25µg/ml and incubated at 30°C for 48 h using OxoPlate reader. The test bacterium was used at various inoculum sizes from $6x10^8$ cfu/ml to $1.2x10^5$ cfu/ml.

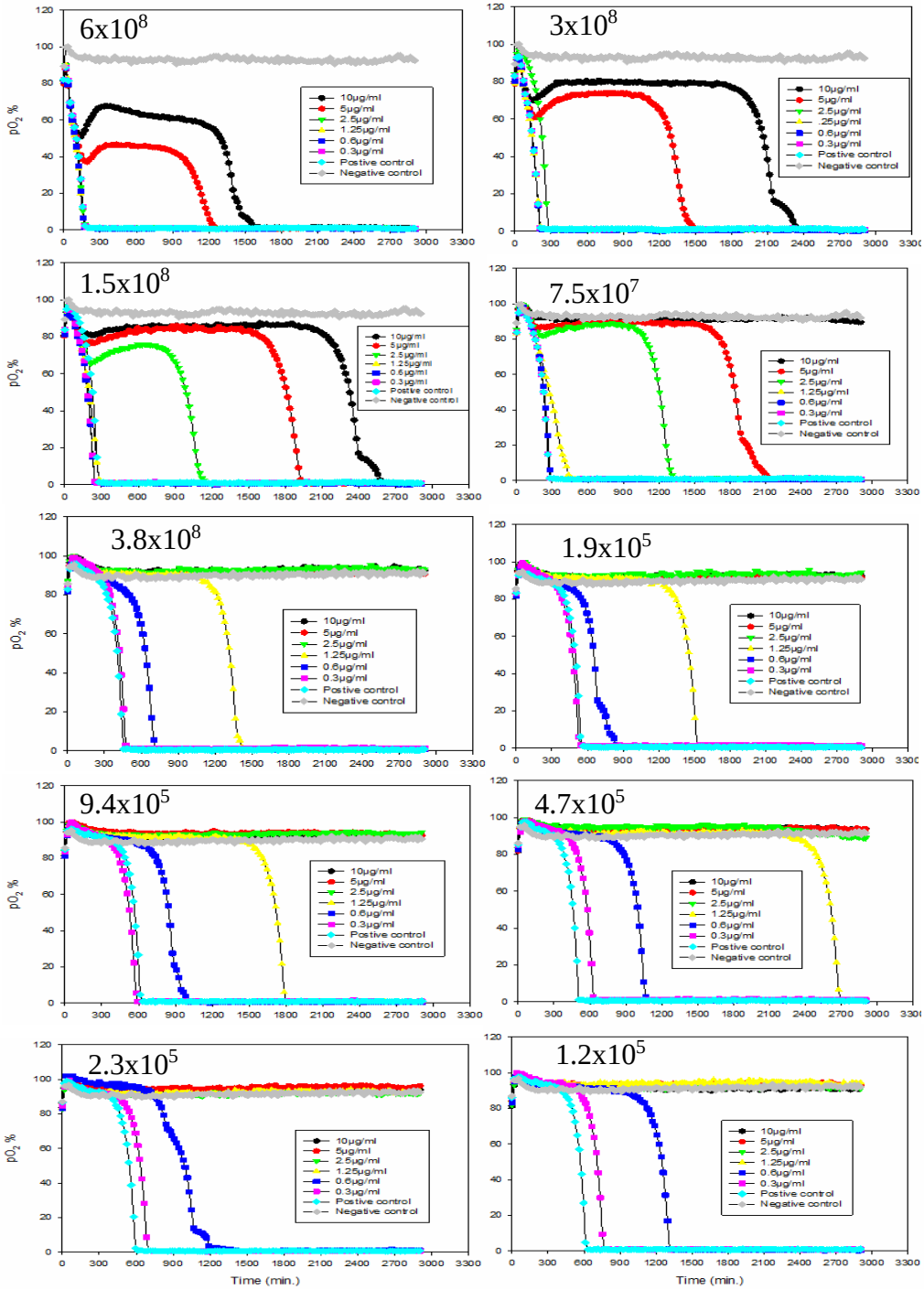


Figure D.2.: Inoculum effect on MIC and MBC of amikacin. Planktonic cells of *P.aeruginosa* were treated with amikacin at different concentrations from 10-0.3µ1g/ml and incubated at 30°C for 48 h using OxoPlate reader. The test bacterium was used at various inoculum sizes from $6x10^8$ cfu/ml to $1.2x10^5$ cfu/ml.

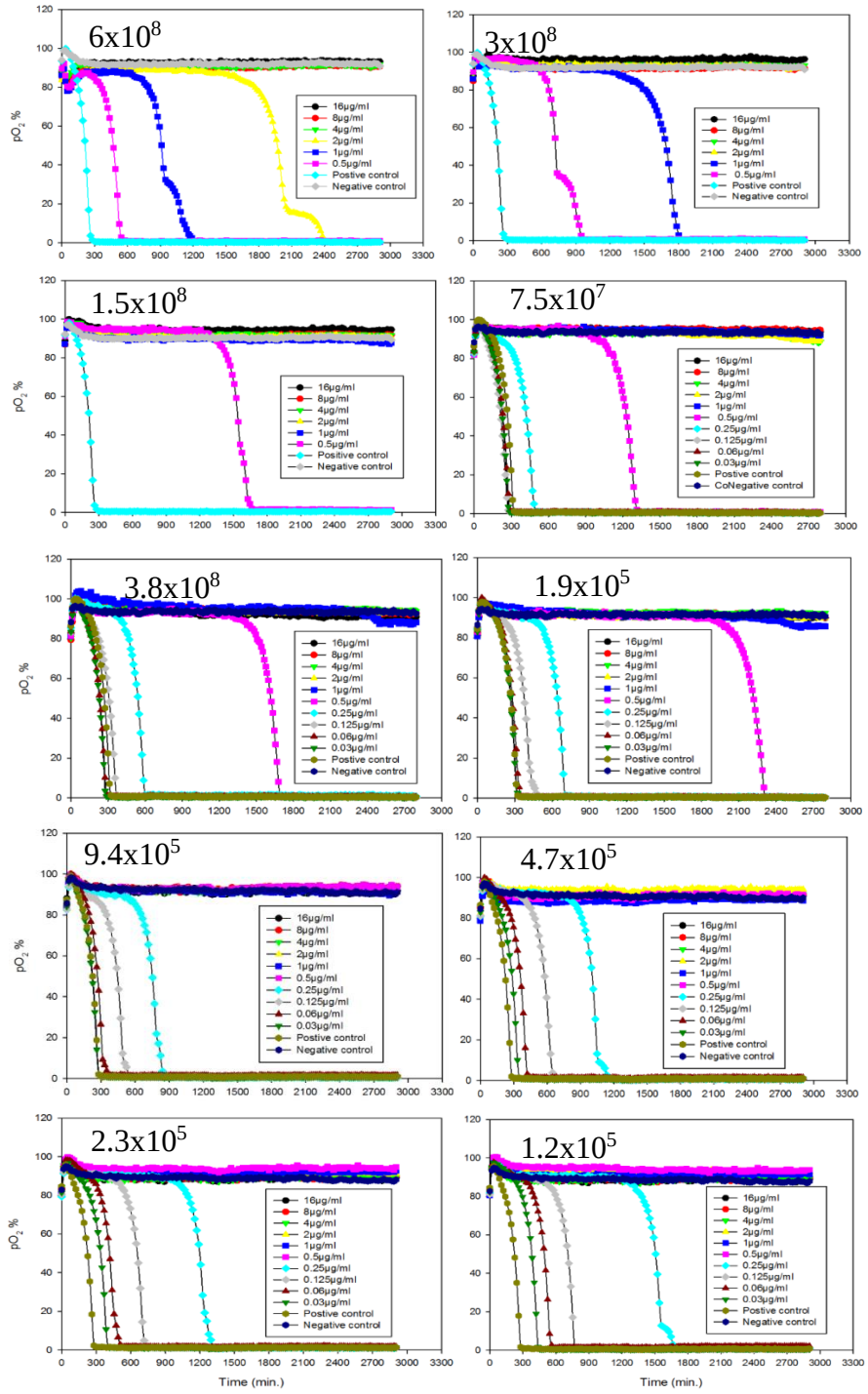


Figure D.3: Inoculum effect on MIC and MBC of colistin. Planktonic cells of *P.aeruginosa* were treated with colistin at different concentrations from 16-0.0.03µg/ml and incubated at 30°C for 48 h using OxoPlate reader. The test bacterium was used at various inoculum sizes from $6x10^8$ cfu/ml to $1.2x10^5$ cfu/ml.

Appendix E: Nutrient limitation and batch mode

Table E.1: Statistical analysis of limiting nutrient concentrations of glucose

mM	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
5	2	.83	.92	.8750	.04500	.06364
10	2	1.17	1.29	1.2300	.06000	.08485
15	2	.50	.50	.5000	.00000	.00000
20	2	1.39	1.65	1.5200	.13000	.18385
25	2	1.41	1.72	1.5650	.15500	.21920
30	2	1.37	1.62	1.4950	.12500	.17678
35	2	1.37	1.58	1.4750	.10500	.14849
40	2	1.33	1.55	1.4400	.11000	.15556
45	2	1.37	1.61	1.4900	.12000	.16971
50	2	1.34	1.58	1.4600	.12000	.16971
Valid N (listwise)	2					

Correlations

		OD	Glucose
OD	Pearson Correlation	1	.999**
	Sig. (2-tailed)		.001
	N	4	4
Glucose	Pearson Correlation	.999	1
	Sig. (2-tailed)	.001	
	N	4	4

**. Correlation is significant at the 0.01 level (2-tailed).

Table E.2: Statistical analysis of limiting nutrient concentrations of nitrate

mM	N	Minimum	Maximum	Mean		Std. Deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
1	2	.23	.23	.2305	.00150	.00212
2	2	.50	.54	.5215	.02150	.03041
3	2	.64	.72	.6785	.03650	.05162
4	2	.87	.89	.8815	.01150	.01626
5	2	.93	.97	.9475	.02250	.03182
Valid N (listwise)						

Correlations

		OD	Nitrate
OD	Pearson Correlation	1	.980**
	Sig. (2-tailed)		.003
	N	5	5
Nitrate	Pearson Correlation	.980	1
	Sig. (2-tailed)	.003	
	N	5	5

**. Correlation is significant at the 0.01 level (2-tailed).

A.

	S	μ	1/S	1/μ
mM	g/l	h-1	g/l	h-1
5	0.9	0.0621	1.111111	16.10306
10	1.8	0.0699	0.555556	14.30615
15	2.7	0.074	0.37037	13.51351
20	3.6	0.0786	0.277778	12.72265
25	4.5	0.078	0.222222	12.82051
30	5.4	0.0778	0.185185	12.85347
35	6.3	0.0622	0.15873	16.07717
40	7.2	0.05335	0.138889	18.74414
45	8.1	0.0509	0.123457	19.64637
50	9	0.0519	0.111111	19.26782

B.

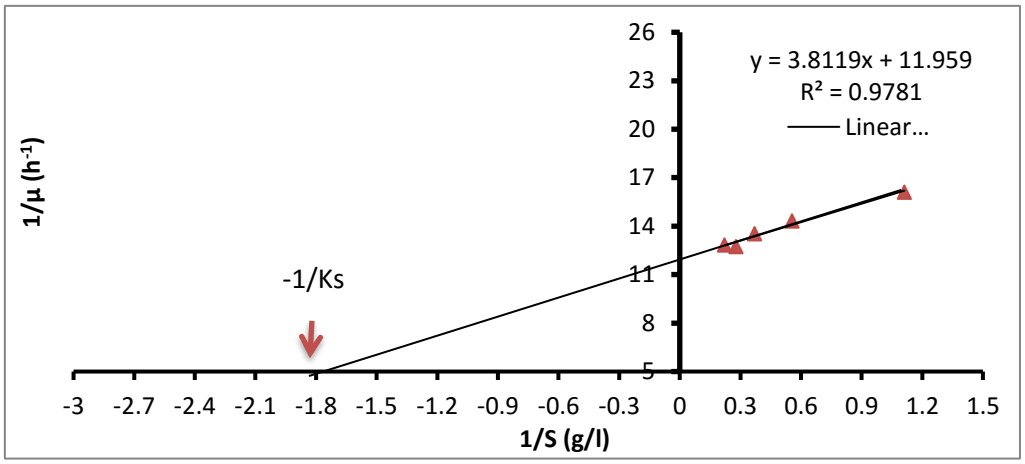


Figure E.1: (A) illustrates the substrate concentration(S) and specific growth rate (μ) to determine the Ks. (B) represents the *Ks* value.

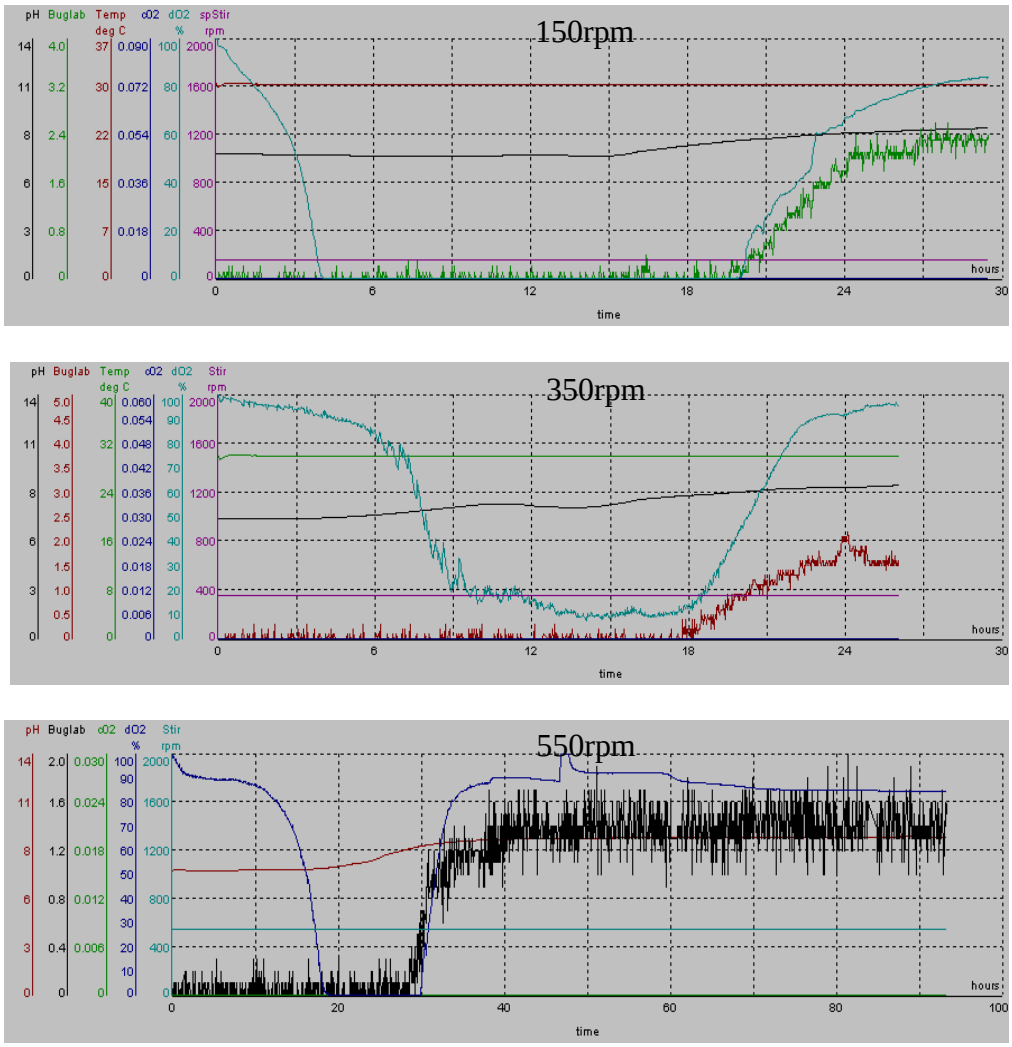


Figure E.2: Data plots of batch modes at different agitation rate.

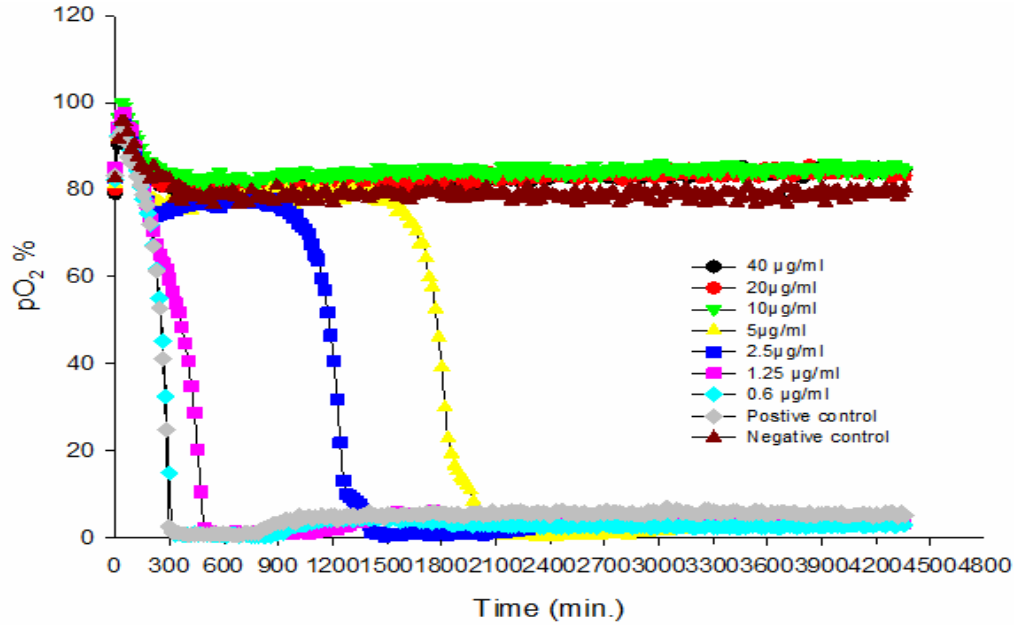


Figure E.3: Antimicrobial susceptibility testing of *P.aeruginosa* during batch mode using MHB. The OD was adjusted to equal the 0.5 MacFarland's standard.

Appendix F: Continues cultures

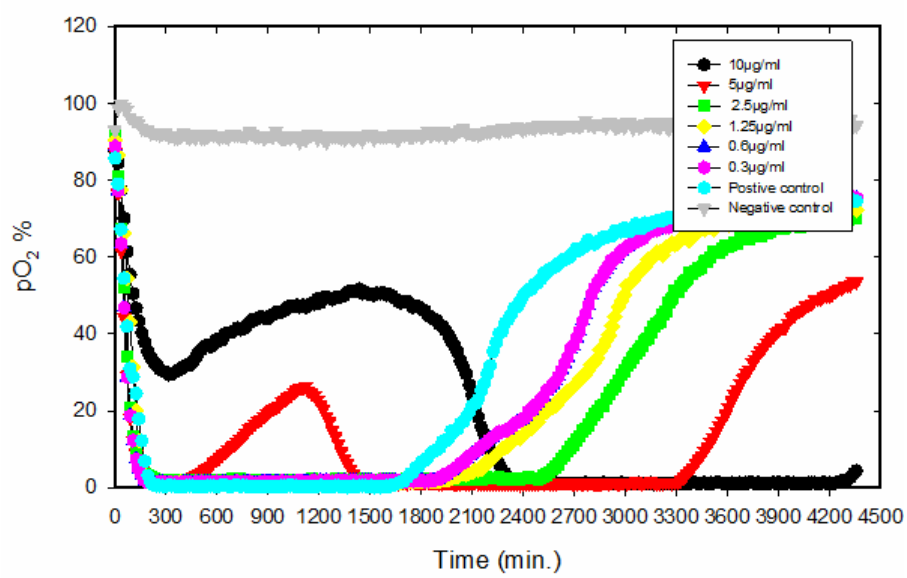


Figure F.1. MIC of *P.aeruginosa* during continues culture before antibiotic introduced using Evan's medium. The density of cells was 0.725 at 625nm. Note: This experiment was done to determine the susceptibility of cells in chemostat before introduce sub-inhibitory concentration.

Table F.1: Measurement of flow rate, doubling time and glucose assay during steady state

D (h^{-1})	F (h^{-1})	td (h)	Glucose (g/l)
0.025	50.4	27.5	0.026466
0.06	120	11.55	0.014809
0.09	198	7	0.088685
0.125	240	5.7	0.076274

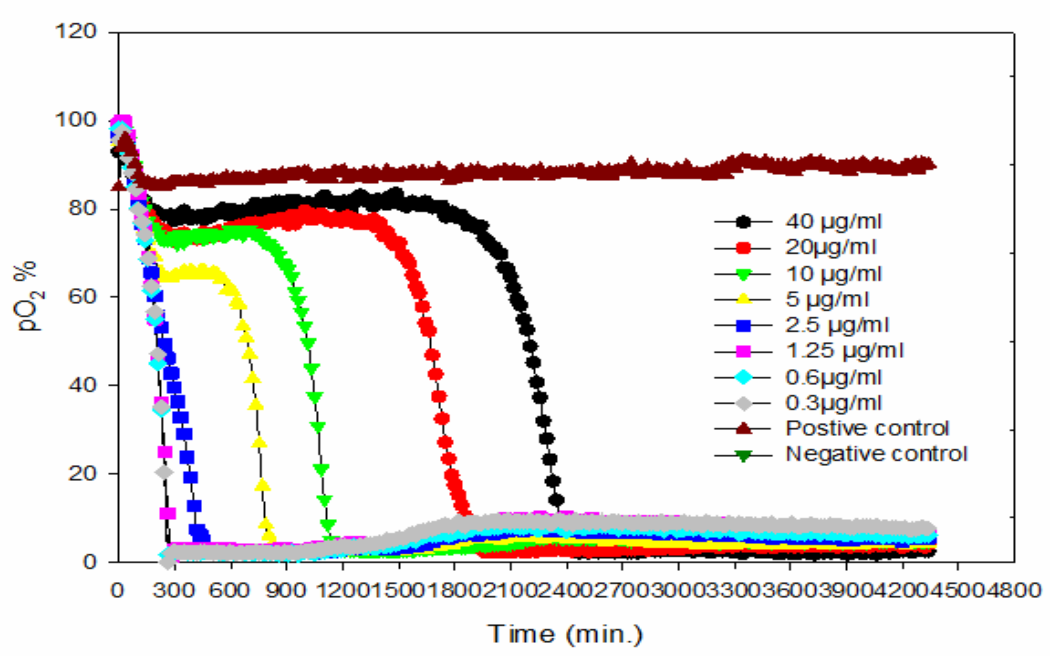


Figure F.2. MIC of amikcain on the population of PA01when grown at 0.025h^{-1} using MHB

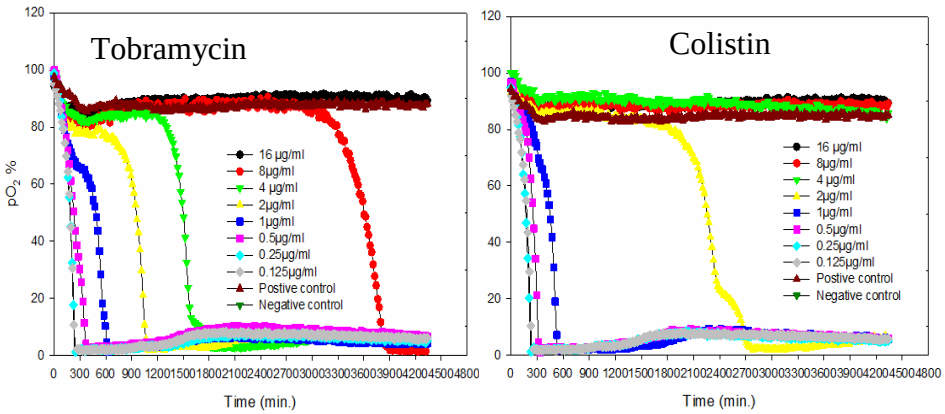


Figure F.3. Adaptive resistance to amikacin crossed low-level resistance to tobramycin and colistin when *P.aeruginosa* grown at 0.025h^{-1}

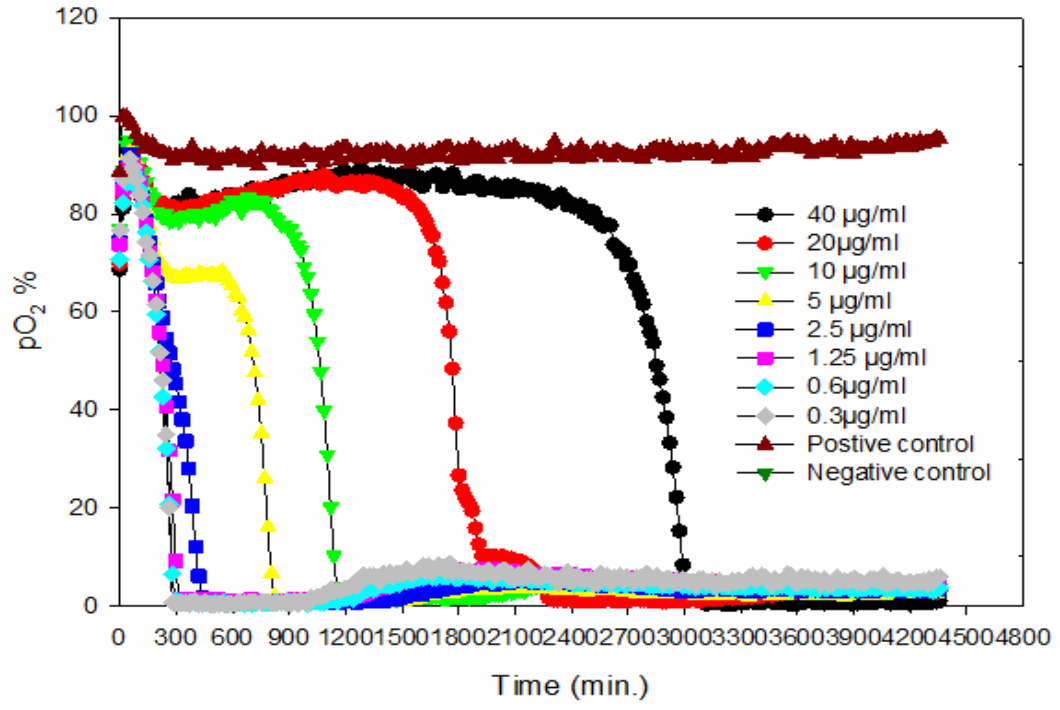


Figure F.4. MIC of amikcain on the population of PA01when grown at 0.06h^{-1} using MHB

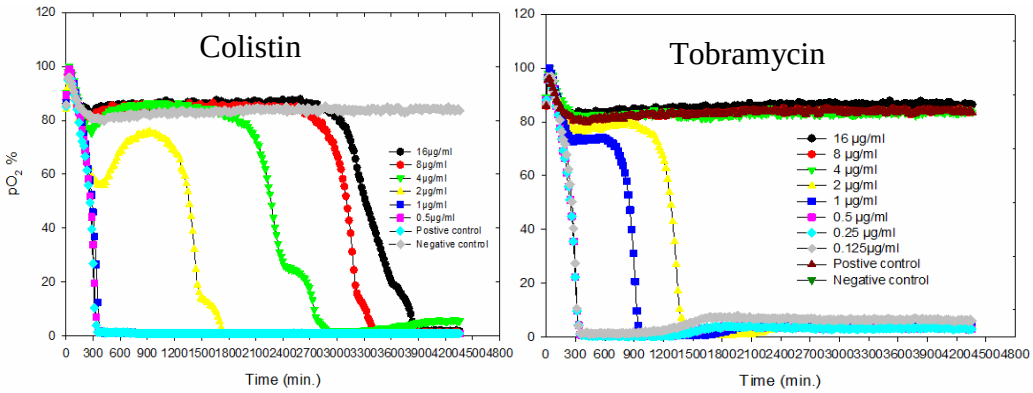


Figure F.5. Adaptive resistance to amikacin crossed low-level resistance to tobramycin and colistin when *P.aeruginosa* grown at 0.06h^{-1}

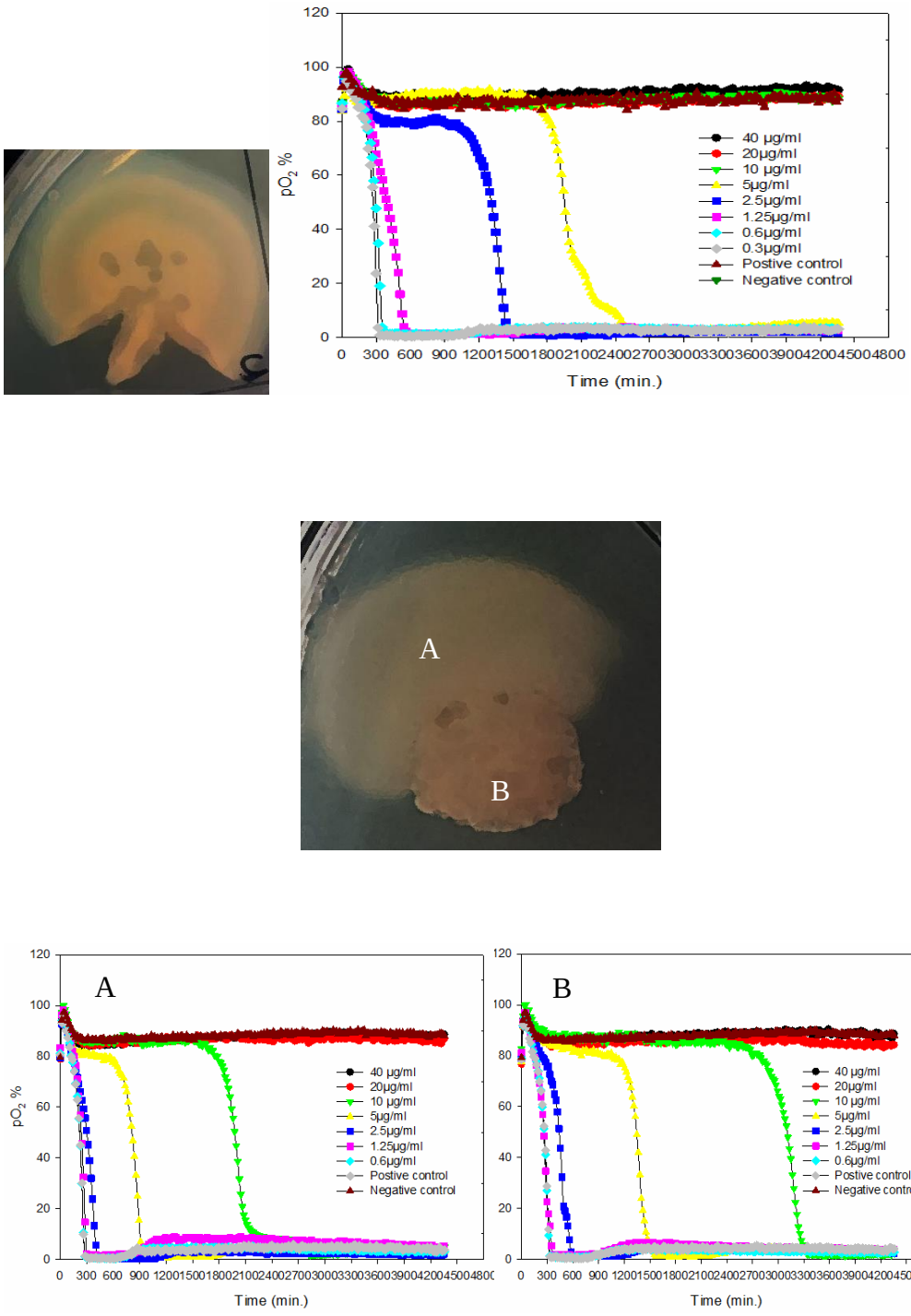


Figure F.6. The MIC and MBC of morphotypes. It had been isolated from chemostat in the presence of amikacin at 0.025$h^{-1}$and incubated at 30^{o}C for 6 days in amikacin-free nutrient agar**.**

A.

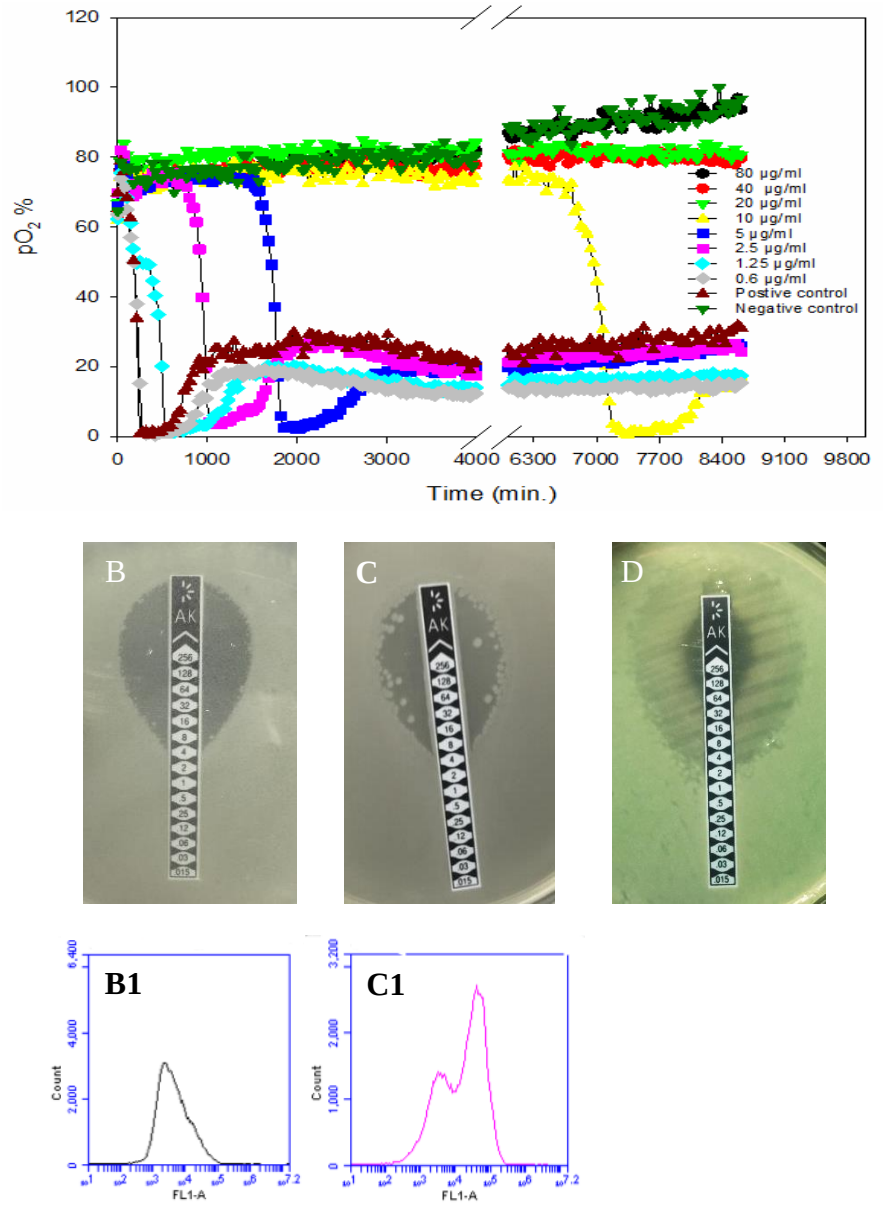
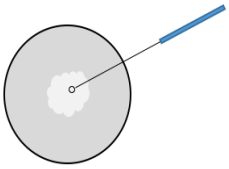
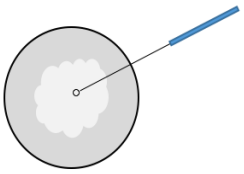


Figure F.7: The MIC and MBC of control culture incubation at 30ºC for 6days to detect survival cells (A). E-test represents the control culture after 3 days (B), survival colonies after 6days incubation (C) and the MIC of survival colonies (D). Histograms show FL1-A, which represent the cell sorting of live cells of culture after 3day (B1) and 6 days(C1) incubation.

3 **Incubation Time (Day)** **6**

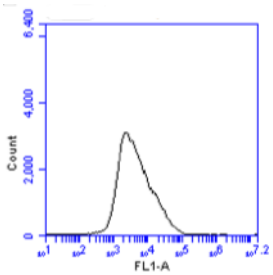


Loop of culture was removed from the centre and edge

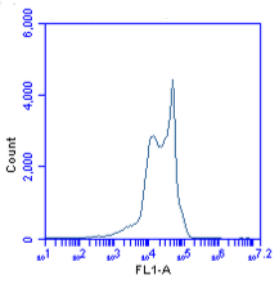


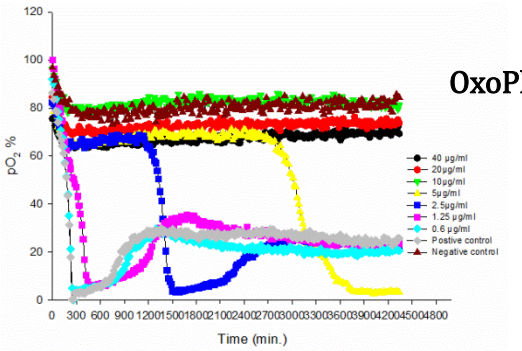
Analysis

A.

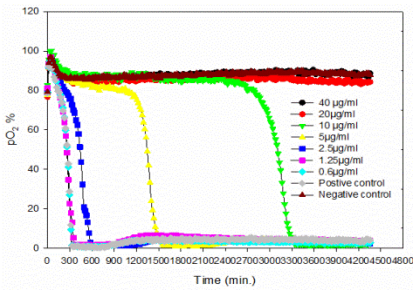


Flowcytometry





OxoPlate



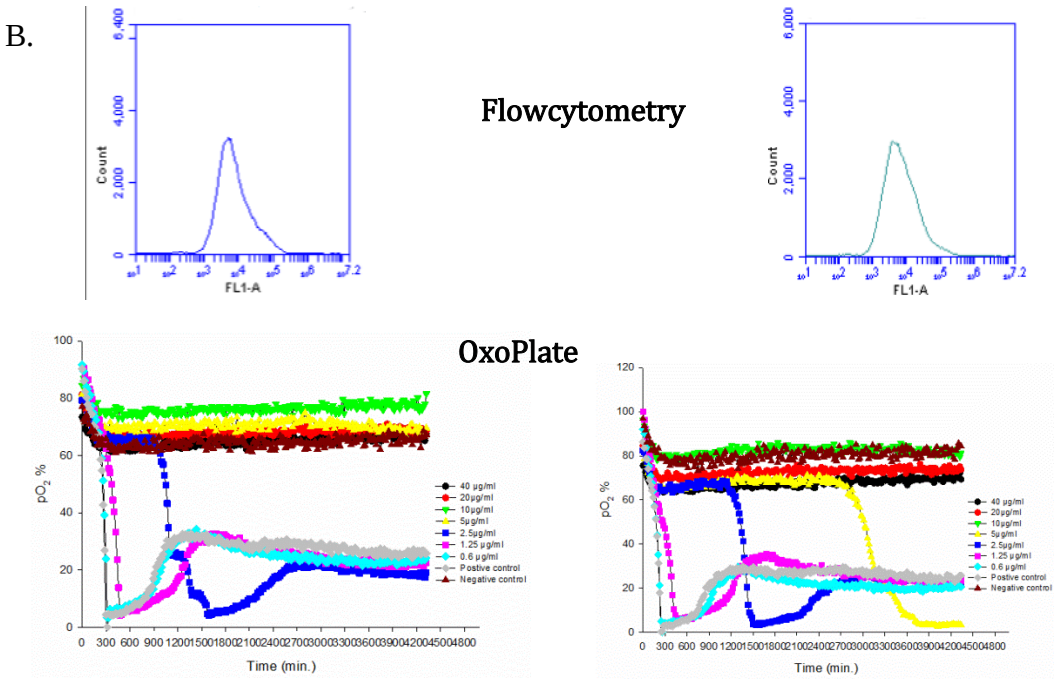


Figure F.8: Effects of growth time of colonies **of** *P.aeruginosa* on the susceptibility to amikacin. It had been isolated from the centre the edge of colony and incubated at 30ºC in amikacin-free nutrient agar (control). OxoPlate output represents the MIC and MBC of colonies incubated for 3 and 6 days. Flowcytometry analysis of different stages of colony growth at centre (A) and edge (B). Histograms show FL1-A which represent the fluorescent parameter of live cells.

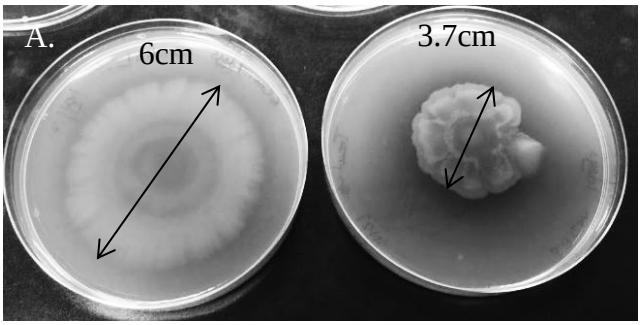


Figure F.9: The colony growth rate on amikacin-free nutrient agar. Bacterial cells were adjusted to equale the 0.5 MacFarland's standard and injected (20µl) in NA. Fast growth was observed in the control culture (A) and slow growth in treated bacterial cells growing in continues culture (B) when incubated for 6 days at 30°C.

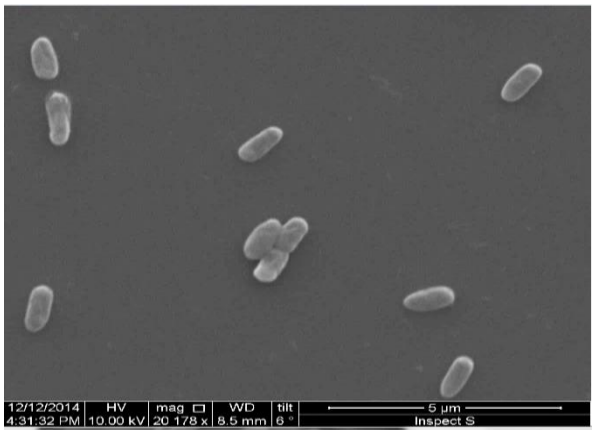


Figure F.10: SEM image showing no extracellular polymeric fibrils presented in the cells derived from the centre of colony of *P.aeruginosa*. It had been isolated from cultures lacking antibiotic (Control) and incubated at 30^{o}C for 3 days.

Appendix G: Poster presentation

Al matrood, W, Evans, K., Smalley, H and Hobbs, G. (2012). Antibiotic resistance: phenotypic changes. A poster presented at the annual Faculty of Science research seminar day. The upper foyer of the Peter Jone Enterprise Centre.

Al matrood, W, Evans, K., Smalley, H and Hobbs, G. (2014). Factors that enhance the ability of *Pseudomonas aeruginosa* to resist the action of antibiotics. A poster presented at the Society for General Microbiology Conference at the Arena and Convention centre. Liverpool, UK. Abstract number LI11/14.

Al matrood, W, Evans, K., Smalley, H and Hobbs, G. (2014). Factors that enhance the ability of *Pseudomonas aeruginosa* to resist the action of antibiotics. A poster presented at the 7th Saudi Students Conference. Edinburgh International Conference Centre, UK.

Al matrood, W, Evans, K., Smalley, H and Hobbs, G. (2015). The effect of divalent cations on the susceptibility of *Pseudomonas aeruginosa* to antibiotics. A poster presented at the 8th Saudi Students Conference. Imperial College London, United Kingdom.

Al matrood, W, Evans, K., Smalley, H and Hobbs, G. (2016). A comparative study of the antimicrobial properties of spices extracts and antimicrobial agents against *Pseudomonas aeruginosa.* A poster presented at The 9th Saudi Student Conference. The International Convention Centre, Birmingham.