Letters

The effect of Some Boron Derivatives on Kanamycin Resistance and Survival of *E. coli* and *P. aeruginosa* in Lake Water*

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Abstract

Objective To study MIC value of 7 boron derivatives namely [Boric acid (H_3BO_3), Anhydrous Borax ($Na_2B_4O_7$), Sodium Borate ($NaBO_2$), Diammonium Tetraborate $(NH_4)_2B_4O_7$, Sodium Perborate ($NaBO_3$), Boron Trioxide (B_2O_3), Potassium Tetraborate ($K_2B_4O_7$)] on *E. coli* and *P. aeruginosa* and their effects on survival of bacteria in lake water and resistance against kanamycin antibiotic.

Methods MIC values of Boron derivatives and antibiotic were studied by broth microdilution method. The effect of boron derivatives on survival of bacteria in lake water were also determined with plate count.

Results Sodium perborate was determined as the most effective substance among the studied substances. Effectiveness increased as temperature increased. *E. coli* was more affected from *P. aeruginosa* in 8 mg/mL sodium perborate concentration in lake water. Moreover, it was determined that MIC value of kanamycin antibiotic decreased 200 times by especially treating *P. aeruginosa* with sodium perborate in lake water. However, it can be stated that this change in resistance did not arise from microorganisms.

Conclusion Sodium perborate solution can be used supportedly in kanamycin antibiotic applications for *P. aeruginosa*. Future studies are necessary to explore the relation between sodium perborate and kanamycin which is effective on *P. aeruginosa* in lake water.

Key words: Boron; Lake water; Kanamycin; *E. coli*; *P. aeruginosa*

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INTRODUCTION

Boron is an element existing abundantly in rocks, soil and water and it exists in amount of 1 mg/L in fresh waters, of 4.6 mg/L in sea and of 100 mg/kg in rocks[1]. Boron acts as non-metal when it reacts with sodium and as metal when it reacts with fluorine. Boron constitutes compounds with sugar, phenol, organic acids and lipids[2]. This element is biologically essential for plants. Although it is quite important for nutrition for plants, its excessive amount is toxic[3]. Moreover, it has very low toxicity on people and it can increase the utility of medicine in diseases such as cancer[4]. Although boron is a necessary for flowering plants, diatoms and some

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types of green algae, it is an essential for the growth of fungi and bacteria[5].

Boron is the main part of intercellular communication signal molecules named as quarum sensing in bacteria[6-7]. Bioluminescent sea bacteria identified AI-2 furanosyl borate diester complex produced as one of 2 autoinducers organizing production of light in response to absence of cells determined in *Vibrio harveyi*[8]. In the future studies, this molecule was determined as signal compound boron containing universal bacterial quarum sensing[9]. Boron isn't an element used in metabolic cases. It was determined that some bacteria synthesized antibiotic containing boron which constitutes their defence systems[10]. Antibiotics named as Boromycin, Aplasmomycin, Borophycin and Tartrolon are synthesized by bacteria and provide their mechanism of action with boron element in their structure. It was determined that they lost their effect when boron element was removed from structure. Boromycin antibiotic is an antibiotic that is synthesized by *Streptomyces antibioticus* and strongly inhibites HIV-1 replication[11-12]. Moreover, it was determined that it inhibited RNA, DNA and protein synthesis and connected with lipoproteins[13].

It was put forth that in carrying out the effect of this antibiotic, boron element in its structure has a role. This antibiotic is boron-centered and it has strong effect on HIV virus implicating boron will have a very important place in novel treatment methods for AIDS. Aplasmomycin is an antibiotic obtained from *Streptomyces griseus* bacteria and showed to have quite importance especially on gram positive bacteria[14-15]. As for Tartralon, it was isolated from *Streptomyces* types and it was determined that is effective on 2 insect types[16]. It is effective on gram positive bacteria, but not on gram negatives[10]. Borophycin is a boron-centered chemical isolated from *Nostoc linckia*[17]. Furthermore, it exists in literature that it is used as eye drops, tooth whiteners, contact lens solutions, antifungal creams,and used as pesticides and disinfectant[18-19]. Based on these, it was showed that antimicrobial features of chemical substances made from boron derivatives can be benefited in the future.

It is estimated that total boron reserves in the world is 1,2 gigatones. 60%-70% of these reserves is in Turkey[19]. Boron derived compounds are used for different purposes in nearly 250 industrial fields such as fiber optics, glass, ceramics, nuclear industry, military, and armoured vehicles, electricity, electronics, computer, construction-cement, metallurgy, energy, automotive, and textile[19-21].

This study was carried out in order to study some boron types which are commercial produced on lives of bacteria when they get mixed into natural life and to explore their utility features and state if there is a change in bacteria's features of resistance against antibiotics.

MATERIAL AND METHODS

Bacteria and MIC Tests of Complexes

The stock solutions of Boron derivatives were prepared with distilled water. The *in vitro* susceptibility tests (MIC tests) were determined by broth dilution carried out in accordance with NCCLS guidelines (NCCLS guidelines 2006)[22]. *E. coli* W3110 and *P. aeruginosa* ATCC27853 were incubated in nutrient agar (Merck) at 37 °C for 24 h. A few colonies were emulsified into 5 mL Nutrient Broth (NB), and adjusted spectrophotometrically to 0.1 absorbance at OD_{600}. The final inoculum was adjusted to $5x10^6$ CFU/mL at 60 mL NB. 1.9 mL resulting suspension was transferred into 1. tube, added 1 mL into other tube, then transferred 100 µL boron derivatives into 1. tube. Samples were serially diluted for 50% and then incubated at 37 °C for 24 h. The same study was repeated after narroving gap. The MIC was defined as the lowest concentration that completely prevented visible growth of the bacteria after overnight incubation at 37 °C. The study were independently repeated for three times and double repeated at the same time**.**

The Effect of Boron Derivatives on Survival of Bacteria in Lake Water

Lake waters were supplied from Porsuk Dam Lake. Fresh lake waters taken for experiments were filtered with whatman filter paper and then sterilized by making auto-claving. The effect of boron derivatives on survival of bacteria was tested at different temperatures (30 °C, 24 °C, and 10 °C), at defined concentration (16 mg/mL for 6 derivatives and 8 mg/mL for $NaBO_3$). Bacteria were incubated in NB (Merck) at 37 °C for 24 h. 1 mL bacterial solution was centrifuged after incubation and washed with lake water for 2 times. Obtained bacterial suspension was used as stock. Bacteria was added into 100 mL filtre-otoclave lake water (final concentration approximately $5x10^6$ CFU/mL) and boron derivatives were transferred into lake water and incubated at different temperature (30 °C, 24 °C, and 10 °C) for 8 h, and samples were serially diluted in ringer solution at several time points and plated into NA. The colonies were counted after incubation at 37 °C for 24 h.

Kanamycin Antibiotic Tests

Bacteria were incubated in nutrient agar (Merck) at 37 °C for 24 h. A few colonies were emulsified into 5 mL Nutrient Broth (NB), and adjusted spectrophotometrically to 0.1 absorbance at OD_{600}. The final inoculum was adjusted to 10^6 CFU/mL at 60 mL NB. Prepared bacterial suspension was poured into test tubes, 32 μg/mL kanamycin antibiotic was poured into the 1st tube and quick dilution was made. Later, 100 μL from lake waters in which there are *E. coli* and *P. aeruginosa* treated with boron derivatives for 4 h were added to each tube. As interesting results were obtained in sodium perborate, lake waters containing this substance were centrifuged and bacteria were washed. In the washed bacteria suspension, 100 μL was added and MIC tests of antibiotic were carried out. Control samples to which no chemical substances were added were compared and whether there is a change in MIC concentration of antibiotic was determined.

RESULTS

MIC Values of Boron Derivatives and Effect of Bacteria on Life in Lake Water

MIC values of boron derivatives used in the study are shown in Table 1. As it can be seen in Table 1, all studied boron derivatives are more effective on *E. coli* than on *P. aeruginosa*. While Boric acid (H_3BO_3) 8 mg/mL, Anhydrous Borax ($Na_2B_4O_7$) 8 mg/mL, Sodium Borate ($NaBO_2$) 4 mg/mL, Diammonium Tetraborate $(NH_4)_2B_4O_7$ 4 mg/mL, Sodium Perborate ($NaBO_3$) 0.5 mg/mL, Boron trioxide (B_2O_3) 4 mg/mL, Potassium tetraborate ($K_2B_4O_7$) 4 mg/mL MIC values were obtained in *E. coli*, 8 mg/mL, 8 mg/mL, 16 mg/mL, 8 mg/mL, 0.5 mg/mL, 4 mg/mL, 16 mg/mL for boron derivatives were obtained in *P. aeruginosa* respectively (Table 1).

At the end of the incubation of bacteria transferred to lake water for 8 h carried out with 6 boron derivatives (16 mg/mL final concentration in lake water), only $NaBO_2$ derivative shows 2 log. decrease in *E. coli* (Table 2). All of the studied substances other than $NaBO_2$ and $NaBO_3$ was more effective on survival of *P. aeruginosa* according to *E. coli* in lake water. As for $NaBO_3$, as it completely inhibited at 1 hour in 16 mg/mL concentration in two bacteria, 8 mg/mL concentration was used (Table 3). It was determined that this substance was also more effective on *E. coli*. All studied boron derivatives are more effective at high temperatures than at low temperatures (Table 2 and 3). Especially, the activity of $NaBO_3$ was highly related with temperature (Table 3). The value of t_{99} was a time that was seen to 2 log. reduction in the number of bacteria according to

Table 1. The Minimal Inhibitory Concentration (MIC) values of Boron derivatives (mg/mL)

Boron Derivatives	*E. coli*	*P. aeruginosa*
Boric acid (H_3BO_3)	8	8
Anhydrous Borax ($Na_2B_4O_7$)	8	8
Sodium metaborate ($NaBO_2$)	4	16
Diamonyum tetraborate $(NH_4)_2B_4O_7$	4	8
Sodium perborate ($NaBO_3$)	0.5	0.5
Boron trioksit (B_2O_3)	4	4
Potassium tetraborate ($K_2B_4O_7$)	4	16

Table 2. The Effect of Boron Derivatives on Survival of *E. coli* and *P. aeruginosa* in Lake Water (16 mg/mL)

Boron Derivatives	Temperature (°C)	*E. coli*			*P. aeruginosa*		
		0 h	4 h	8 h	0 h	4 h	8 h
Control	30	6.9	6.85	6.86	6.65	6.68	6.64
	24	6.9	6.86	6.88	6.65	6.65	6.66
	10	6.9	6.87	6.85	6.65	6.66	6.68
H_3BO_3	30	6.9	6.75	6.53	6.65	6.26	5.95
	24	6.9	6.75	6.55	6.65	6.05	5.56
	10	6.9	6.83	6.70	6.65	6.67	6.71
$Na_2B_4O_7$	37	6.9	6.66	6.65	6.65	6.08	5.96
	24	6.9	6.98	6.88	6.65	6.62	6.68
	10	6.9	6.85	6.80	6.65	6.60	6.45
$NaBO_2$	30	6.9	5.54	4.90	6.65	6.35	6.05
	24	6.9	6.15	5.2	6.65	6.60	6.58
	10	6.9	6.47	5.59	6.65	6.63	6.65
$(NH_4)_2B_4O_7$	30	6.9	6.78	6.46	6.65	6.04	5.88
	24	6.9	6.89	6.90	6.65	6.58	6.55
	10	6.9	6.80	6.70	6.65	6.60	6.60
B_2O_3	30	6.9	6.72	6.85	6.65	5.85	5.88
	24	6.9	6.92	6.88	6.65	6.58	6.57
	10	6.9	6.80	6.78	6.65	6.60	6.56
$K_2B_4O_7$	30	6.9	6.85	6.53	6.65	6.11	5.95
	24	6.9	6.70	6.26	6.65	6.68	6.70
	10	6.9	6.80	6.76	6.65	6.59	6.62

initial bacterial count, and are given in Table 3. When effect of $NaBO_3$ on survival of *E. coli* in lake water was examined, t_{99} value was 16 minutes in 30 °C and 45 min in 10 °C. According to t_{99} value, *P. aeruginosa* was 2 log. decrease at 60 min in 30 °C, at 180 min in 10 °C (Table 3).

Table 3. The Effect of Sodium Perborate ($NaBO_3$) on Survival of *E. coli* and *P. aeruginosa* in Lake Water (8 mg/mL final concentration)

	Temperature(°C)	0 h	30 min	1 h	2 h	3 h	4 h	t_{99} (min)
E. coli	30	6.95	3.10	2.01	0.00	0.00	0.00	16
	24	6.95	4.95	3.10	1.13	0.00	0.00	30
	10	6.95	5.90	4.30	1.58	0.00	0.00	45
P. aeruginosa	30	6.65	5.38	4.65	2.69	1.27	0.00	60
	24	6.65	5.95	5.20	4.30	2.79	1.25	90
	10	6.65	6.22	5.79	5.25	4.66	3.75	180

Effect of Boron Derivatives on MIC Value of Kanamycin Antibitocs

MIC value of control samples with MIC value of kanamycin antibiotic of bacteria treated with boron derivatives in lake water for 4 h were compared. Moreover, bacteria count was carried out after 4 h. The results are shown in Table 4 and 5.

MIC values of samples of *E. coli* which weren't treated with a substance was determined as 0.5-1 µg/mL at different temperature. It was determined that *E. coli* changed in its sensitivity against kanamycin antibiotic except boric acid (H_3BO_3) when it was treated with boron derivatives in lake water in different temperatures. However, this change wasn't in the direction of gaining resistance, on the contrary, it increased sensitivity of bacteria against antibiotics. The remarkable reduction in resistance was found at high temperature (30 °C). The changes at the other temperatures is not important. Hence, the effectiveness of matter increased on cell due to the high metabolisms of bacteria in 30 °C. This effectiveness may be thought to reduce resistance in bacteria. While Boric acid (H_3BO_3) was found to have same values with control group, other substances caused increase in sensitivity at the ranges of changing about 2-4 times. Although there was a 4 times decrease in $NaBO_3$ in 30 °C, it is thought that this decrease is due to the scarcity of bacteria count (Table 4A- $NaBO_3$).

In the study with *P. aeruginosa*, a remarkable result was a decrease of 200 times in MIC value of kanamycin antibiotic in the samples treated with $NaBO_3$ compared to control and no other remarkable changes were found in other substances. It was determined that when *P. aeruginosa* treated with $NaBO_3$ in lake water was taken directly and transferred to NB culture, this effect exists and when bacteria obtained as pellet by centrifuging lake water was washed and transferred to NB, MIC value was the same as that in control group. Based on all above mentioned, it is thought that MIC value of *P. aeruginosa* decreased 200 times not because of a change in bacteria treated with substance, but because of a relation between perborate in lake water and kanamycin in NB.

Table 4. The Effect on the Survival of *E. coli* of Boron Derivatives in Lake Water (cfu/mL) (A) and The Effect on MIC Value of Kanamycin Antibiotic (µg/mL)(B)

A		Control	$NaBO_3$ 1 mg/mL	H_3BO_3 4 mg/mL	$Na_2B_4O_5$ 4 mg/mL	$NaBO_2$ 4 mg/mL	$(NH_4)_2B_4O_7$ 4 mg/mL	B_2O_3 4 mg/mL	$K_2B_4O_7$ 4 mg/mL
	0 h	6.52	6.52	6.52	6.52	6.52	6.52	6.52	6.52
30 °C	4 h	6.49	4.53	6.56	6.57	6.48	6.66	6.52	6.40
24 °C	4 h	6.54	5.60	6.62	6.40	6.30	6.36	6.28	6.69
10 °C	4 h	6.50	6.01	6.58	6.38	6.26	6.46	6.15	6.52
B		**Control**	**$NaBO_3$**	**H_3BO_3**	**$Na_2B_4O_5$**	**$NaBO_2$**	**$(NH_4)_2B_4O_7$**	**B_2O_3**	**$K_2B_4O_7$**
30 °C		1.0	0.125	1.0	0.25	0.25	0.25	0.25	0.25
24 °C		0.5	0.25	0.5	0.25	0.25	0.50	0.50	0.25
10 °C		1.0	0.50	1.0	0.25	0.50	0.50	0.50	0.50

Table 5. The effect on the survival of *P. aeruginosa* of boron derivatives in lake water (cfu/mL) (A) and The effect on MIC value of Kanamycin antibiotic (μg/mL) (B)

A		Control	$NaBO_3$ 1 mg/mL	H_3BO_3 4 mg/mL	$Na_2B_4O_5$ 4 mg/mL	$NaBO_2$ 4 mg/mL	$(NH_4)_2B_4O_7$ 4 mg/mL	B_2O_3 4 mg/mL	$K_2B_4O_7$ 4 mg/mL
	0 h	6.23	6.23	6.23	6.23	6.23	6.23	6.23	6.23
30 ˚C	4 h	6.14	5.57	5.83	5.92	6.04	5.90	5.86	5.84
24 ˚C	4 h	6.18	5.76	6.20	6.20	6.28	6.26	6.23	6.15
10 ˚C	4 h	6.28	6.08	5.91	5.91	6.32	6.08	6.18	6.38

B	Control	$NaBO_3$	[a]$NaBO_3$	H_3BO_3	$Na_2B_4O_5$	$NaBO_2$	$(NH_4)_2B_4O_7$	B_2O_3	$K_2B_4O_7$
30 ˚C	8	0.039	8	8	8	8	8	8	8
24 ˚C	8	0.039	8	8	16	16	16	8	8
10 ˚C	8	0.039	8	8	16	16	8	16	16

Note. [a]Lake water were centrifuged and Bacteria were washed. Other lake waters did not centrifuged.

DISCUSSION

It is known that chemical substances intervened into nature cause severe problems in lives of bacteria. Boron compounds enter the nature by laundry products, irrigation drain water, fertilizers, boron mineral processing and other industrial sectors. This chemical substances cause serious problems in survival of bacteria in aquatic environments. Also, these substances sometimes change antibiotic resistance values of bacteria. This situation causes hazardous results in terms of public health. There are some studies in literature about the effect of different boron derived substances on bacteria. However, there aren't so many studies showing their effect on both survival and antibiotic resistance of bacteria in natural environments.

It was put forth that boron products are effective on microorganisms and can be used as antiseptic and disinfectant. It was seen that diazaborine is active against bacteria and malaria[23-24]. Moreover, borate complexes occuring naturally can be used as topical antibiotic[24-25]. It was shown that compounds containing B-N have anticancerous, hypolipidaemic and antifungal activity[26]. Oxazaborolidine may serve as novel agents for affecting oral biofilm formation[27]. Furthermore, Bailey *et al.,* (1980) determined that borinic acid and diazoborine type agents possess severe antibacterial activity[28]. In our study, While sodium perborate (8 mg/mL) has more effect on survival of *E. coli* than it has on *P. aeruginosa*, other studied boron derivatives (16 mg/mL) have more effect on the survival of *P. aeruginosa* than they have on *E. coli*. As shown in the literature[29], boron derivatives are substances to effect at high concentration.

It was determined that enzymes such as protease intervened into inhibition rather than borate and boronate complexes[30]. Tetrahedral complex occurs between boron atom and serine hydroxyl group. Furthermore, simple borates are used as protease stabilisers in detergents[31]. Boric acid has a very low LD_{50} value. Therefore, putting boron in molecules similar to medicines is a new area for medicine researchers[24].

According to Pankey and Sabath (2004), a compound is defined as bactericidal if its effects a 3-log or greater decrease in bacterial cell density after 24 h of incubation[32]. Based on this , sodium perborate has bactericidal effect for the two bacteria. Sodium perborate is a compound that consists of 16% active oxygen in monohydrate structure. Oxygen in its structure occurs only in water in 45 °C. Therefore, while stock of this substance was prepared, it was heated below 40 °C and solubilized. If sodium perborate is melted in water, it releases hydrogen peroxide. Features of aqueous solution are practically similar with those of hydrogen peroxide solution. According to this, sodium perborate is considered as solid phase of hydrogen peroxide used as powerful oxidant in several industries including detergent industry. Anhydrous sodium perborate ($NaBO_3$) is used as additive in the production of cleaning materials, as raw material in the production of oxygen, and also used in medicine and dentistry because of its oxidizing feature. Sodium perborate is used as active oxygen welding in several detergents. Moreover, it is used as contact lens solution and disinfectant because of its tooth whitening and antiseptic features[18,33-34]. It has whitening featute which isn't oxidative. It causes little damage on paint and textile products as whitener than sodium hypochloride do. Sodium perborate deoxygenates in temperatures higher than 60 degrees. Therefore, it was solubilized in 40 degrees and its stocks were prepared. Summers et al*.,* (1978) studied on the effects of $Na_2B_4O_7$ and $NaBO_2$ on *P. aeruginosa* and roles of plasmids in resistance[35]. They stated that plasmids play a role in resistance of *P. aeruginosa* against borate and metaborate. 5 mmol/L and 10 mmol/L were determined as MIC value for borate and metaborate respectively[35]. Bacteria are relatively tolerant towards boron. The effect concentration of Boron ranges between 8 and 340 mg boron/litre in bacterial species. It was determined that toxic dose of sodium tetraborate was as 7.6 mg B/L in *P. putida*[29]. $NaBO_3$ may be used in aquatic environments such as lake water, but these concentrations of $NaBO_3$ must be worked with toxicology tests on plants and animals in aquatic environments.

Several factors affect lives of bacteria in aquatic environments. These factors might be such as starvation, temperature, pH and toxical chemical substances[36-37]. It was also determined that these environmental factors changed antibiotic resistance values. It was determined that there were changes in antibiotic resistance in bacteria exposed to starvation stress due to nutritional deficiency. It was determined in a study, that there was a severe decrease in sensitivity of *E. coli* and *S. enteretidis* against antibiotics. They determined that MIC values of bacteria exposed to starvation for 60 days decreased 2 times for rifampicin, gentamicin, ofloxacin and 3 times for ceftazidime[38]. Özkanca et al*.,* determined that antibiotic resistance in *E. coli* increased in chemicals treated with hydrogen peroxide, toluene, ethanol and nitrobenzene in sea water[39]. Similar trends were observed both in pretreated cells and in those surviving in the presence of chemical treatments. However, *S. enteritidis* cells showed a more marked increase in resistance in the presence of chemicals than pretreated cells[39]. Similarly, Munro et al*.,* stated that life strategies developed by *E. coli* exposed to sea water and waste water mixture changed antibiotic resistance sensitivity[40]. It is important for public health that life abilities and defence mechanisms of bacteria in negative environmental conditions are determined and resistance features of human and animal pathogens that can intensively intervene into aquatic environments are put forth.

In our study, it was shown that there was a severe difference between bacteria samples washed by centrifuged and bacteria samples added without being washed. This difference shows a situation not arising from a change occuring in bacteria. Therefore, when MIC values are examined by moving bacteria away from lake water, same values with control samples are obtained. Therefore, it is seen that a new situation occuring as a result of interaction of sodium perborate coming from lake water and kanamycin antibiotic causes a very effective result and this result is effective only on *P. aeruginosa*. According to these results, sodium perborate solution can be used supportedly in kanamycin antibiotic applications for *P. aeruginosa*. This study will provide important data for developing methods or chemical subtances to be used in future studies for treatment-purposes for *P. aeruginosa* which is a quite hazardous bacterium in terms of hospital infections.

In this study, effects of boron derivatives which intervene into aquatic environments such as lake water on survivals of bacteria and changes in the features of resistance against antibiotic were put forth. It was determined that kanamycin and sodium perborate are quite effective on *P. aeruginosa* and Kanamycin antibiotic decreased MIC value nearly 200 times. The mechanisms need further exploration. This study also represents the influence of boron derivatives on survival of bacteria in aquatic habitats such as lake water.

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