

Study of changes in bacterial and viral abundance in formaldehyde- fixed water samples by epifluorescence microscopy

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Abstract

Accurate measurement of bacterial and viral abundance in coastal-marine environments is important to understand the dynamics of microbial communities in these ecosystems. In this study, the effect of formaldehyde preservation on the abundance of bacteria and viruses in water samples from Cochin Backwater was determined by SYBR Green I staining and epifluorescence microscopy. The counts were determined for 45 days in samples fixed with 1-6% formaldehyde. The results suggest rapid decline in counts of bacteria and viruses in samples preserved in formaldehyde and the decline increased with increase in the final concentration of formaldehyde in the sample. The initial bacterial and viral counts in the sampling site were 5.2×10^6 /ml and 3.9×10^7 /ml respectively. The bacterial count dropped by 5.1% , 53.3%, 55.7% and 85% after 1 day, 7 days, 15 days and 45 days respectively in sample fixed with 1% formaldehyde. The decline in viral counts was higher, being 32.4%, 47.9%, 68.1% and 93% after 1, 7, 15 and 45 days respectively. Storage of fixed samples at -20°C did not halt the decline in microbial counts suggesting that irrespective of storage temperature, formaldehyde-fixed samples lead to underestimation of microbial counts.

Introduction

Bacteria and viruses play vital roles in the productivity of marine and estuarine systems. Though bacteria are well known to be key constituents of aquatic ecosystems, the importance of viral communities in food web has only been realized recently (Fuhrman 1999). Viruses are the most abundant entities in natural waters in comparison to their bacterial counterparts ((Bergh et al. 1989; Proctor et al. 1990; Suttle et al. 1990). Viral counts typically range from 10^4 /ml in oligotrophic waters to 10^8 /ml in eutrophic waters (Wommack and Colwell 2000). A vast proportion of marine viruses are phages that kill autotrophic and heterotrophic bacteria thus playing a key role in the structure and function of aquatic food web (Wilhelm and Suttle 1999).

Quantification of viral biomass in marine aquatic systems has become important owing to the realization of ecological roles played by these microorganisms in geochemical cycling of carbon and nutrients (Wilhelm and Suttle 2000). Quantification is done by plaque assay, most probable number (MPN) technique, epifluorescence microscopy, flow cytometry and transmission electron microscopy (TEM) (Marie et al. 1999; Suttle 2000; Brussaard 2004; Ananthanarayan and Paniker 2005; Patel et al. 2007). Epifluorescence microscopy is considered as the most reliable and accurate method for the estimation of viral abundance, comparable with flow cytometry. Both these techniques make use of fluorescent nucleic acid dyes like SYBR Green I (Noble and Fuhrman 1998) or SYBR Gold (Chen et al. 2001) and Yo-Pro-1 (Hennes and Suttle 1995). For this, the water samples are preserved in solutions of formaldehyde or glutaraldehyde followed by storage at 4°C. Studies have shown that the counts decrease with increase in the duration of storage (Gundersen et al. 1996; Wen et al. 2004). In the study reported here, we analyzed the abundance of bacteria and viruses in a fixed station in Cochin backwater by epifluorescence microscopy and determined the effect of different concentrations of formaldehyde on the abundance of bacteria and viruses in preserved water samples.

Materials and Methods

Sample collection and preservation

Triplicate water samples were collected in sterile containers (1000 ml) from Indian Oil Corporation Ltd. (IOCL) jetty, Cochin and transported on ice to the laboratory and processed for analysis within an hour of collection. From each water sample, 100 ml was dispensed into each of the six bottles and fixed with various final concentrations (1%, 2%, 3%, 4%, 5%, and 6%) of formaldehyde and stored at 4°C. Immediately after fixation with formalin, 1 ml from each of these bottles was filtered and stained with SYBR Green I using the protocol described below. The samples were enumerated daily from the day 1 to the day 7. From the 7th day, enumeration was done every 5th day up to the 30th day. The final count was taken on the 45th day. In a separate treatment, one set of sample was preserved at -20°C, without and with formaldehyde at 1% and 2% final concentrations and the counts were taken on every 5th day till the 45th day.

Staining protocol and enumeration of bacteria and viruses

Water samples were stained using SYBR green I fluorescent dye (Invitrogen, CA, USA) as previously described (Noble and Fuhrman 1998). Briefly, 1 ml of the sample was filtered through a 0.02 µm pore-size (25mm diameter) anodisc filter backed by 0.8 µm cellulose mixed ester membrane (Millipore) under 20 Kpa vacuum. The membrane was filtered to dryness and was removed with forceps with vacuum still on. SYBR green I was diluted 1:10 by mixing 2.5 µl of 10% SYBR green working solution with 97.5 µl of 0.02 µl filtered, sterile deionized water on the bottom of a sterile plastic petridish. The anodisc filter as placed on the SYBR green solution in the petriplate, with filtered side containing bacteria and viruses upwards and incubated in the dark for 15 min. After incubation, the filter was transferred to a clean glass slide and kept over a cover slip containing 30 µl antifading solution [50% glycerol, 50% phosphate buffered saline (PBS, 0.05 M Na₂HPO₄, 0.85% NaCl, pH 7.5), 0.1% pphenylenediamine]. The slides were used either immediately for counting or stored at -20°C until enumeration. For each filter, 10-20 fields were selected randomly and a total of >200 viruses and >200 bacteria were counted at 1000× with an Olympus BX 41 epifluorescence microscope under blue excitation.

Results and discussion

Precise estimation of bacterial and viral communities is a prerequisite in coastal-marine productivity studies. Enumeration of bacteria and viruses by epifluorescent microscopy (EFM) is routinely done using formaldehyde-preserved samples. SYBR Green I, a cyanine-based fluorescent dye is preferred for enumeration of viruses in epifluorescence microscopy and flowcytometry owing to its bright, stable fluorescence, and high binding affinity to double stranded DNA and RNA (Noble and Fuhrman 1998; Marie et al. 1999; Patel et al. 2007). However, several factors are known to influence the accuracy of enumeration, the most important being rapid decay of bacteria and viruses in preserved samples (Brussaard 2004; Wen et al. 2004; Shibata et al. 2006). Formaldehyde fixation results in rapid decrease of viral counts by $16.4\% \pm 5.2\%$ within 1 h, by 20 to 35% within 4 h and by 72% after 16 days of storage at room temperature (Wen et al. 2004). This decay of bacteria and virus may also depend on the temperature of storage and intrinsic characteristics of water itself, such as the pH, salinity and the organic matter.

The objective of this study was to determine the effect of preservation of water samples in various concentrations of formalin and at different temperatures of storage. This study is important prior to a large special-scale pilot study on the bacterial and viral biomass in Arabian Sea which requires collection and analysis of multiple sea water samples. Estimation of bacterial and viral numbers was done by SYBR green I staining followed by epifluorescence microscopy for every day for 7 days initially, followed by enumeration at 5 days interval until the 45th day. Figures 1 and 2 show the trend in viral and bacterial counts in formalin fixed samples stored at 4°C. In water sample fixed with 1% formalin, viral count was 3.9×10^7 /ml on the first day and the count decreased to 2.99×10^6 cells/ml on the 45th day. The decline in the count was 47.9% on the 7th day, 68% on the 15th day and 93% on 45th day (Table 1). The viral decay was rapid that started immediately after the fixation of the sample. The initial counts of viruses decreased with increase in the formaldehyde concentrations. It must be noted that initial enumeration on day 1 was done within 30 min of fixation of samples. Time course experiments suggested that the decay was rapid in the first 24 h being 32.4%, 28.3%, 27.6%, 19.6%, 24.7% and 21.6% in samples fixed with 1%, 2%, 3%, 4%, 5% and 6% formaldehyde respectively. The corresponding values on the 7th day were 47.9%, 47%, 41.7%, 34.1%, 35.3% and 39.1% respectively. By 20th day, the viral counts had declined by approximately one log unit or by about 75% in all the

samples. By 45th day, the viral counts in all the fixed samples declined by 89-93%. Our results further suggest that preservation of formalin fixed samples at -20°C does not arrest or reduce the viral decay (Table 1) and the decline in counts was similar to the formalin preserved samples stored at 4°C.

The initial (day 1) bacterial count in the sample fixed with 1% formalin was 5.2×10^6 /ml and by 45th day, the count had reduced to 7.7×10^5 cells/ml, a reduction of 85.3% of the initial count (Table 2). In 2% formalin-fixed sample, the initial bacterial count was 6.62×10^6 cells/ml. The count dropped by 36% in the first 15 days and was further reduced to 36% and 89.1% by the end of 30th and 45th day respectively. The initial bacterial count in 3% formalin was 6.5×10^6 cells/ml. The counts reduced to 78.7% and 89.5% after 30 days and 45 days of incubation. The percentage reduction of bacterial count in 4% formalin was about 77.7% on 30th day and by 45th day there was a sharp decrease in count to about 88%. In 5% formalin the initial bacterial count was 4.99×10^6 cells/ml and the percentage decrease by 30th day was about 79.5% and 87% by the 45th day. The bacterial count using 6% formalin in the first day was 4.36×10^6 cells/ml and the rate of reduction was 69% by the 30th day and 89% by the 45th day (Table 2). The decrease in bacterial counts during the early part of storage is rapid (Table 2). This phenomenon has been previously observed by other investigators in formaldehyde preserved samples stored at 4°C (Shibata et al. 2006). In another study, 41 to 48% of the original concentration of bacteria was lost due to the cell adhering to the wall of the sample containers (Turley and Hughes 1992). In the same study, 27 to 51% of observed loss of bacterial cells could not be accounted for. Enzymatic breakdown, such as the proteolytic activity may be a major cause of the loss of bacteria in preserved samples (Fahimi and Drochmans 1968; Kolb- Bachofen 1977). In addition, the chemical reaction of aldehyde with the components of cells could be responsible for the decay of bacteria. This is evident from higher decay rates in samples preserved in higher concentrations of formaldehyde. In addition, storage of samples at 4°C does not help to improve the preservation or reduce the decay. A previous study has recorded 34% decline in bacterial counts within 7 days in samples preserved at 4°C (Shibata et al. 2006).

In a separate experiment, samples preserved without and with 1% and 2 % formalin and preserved at -20°C were subjected to enumeration (Tables 1 & 2). Viral and bacterial counts were taken on every 5th day till the 30th day and on the 45th day. In the case of samples preserved with 1% formalin, viral counts

decreased by 59% on the 5th day, 62% on the 10th day and 80% on 45th day. Bacterial counts decreased by 42%, 51 %, 63% and 82% on 5th, 10th, 20th and 45th day respectively. In the case of samples fixed with 2% formalin, stored at -20°C, the viral counts decreased by 37 % on 5th day to 82% on 25th day to 93% on 45th day. Bacterial counts decreased by 30% on 5th day, to 87% on the 45th day. Samples stored at -20°C without formalin showed a sharp decrease of 83% in their viral counts by the 45th day, while the bacterial counts decreased by 87% on the 45th day. According to Gundersen et al (1996) bacterial numbers in samples preserved with gluteraldehyde, quick frozen and stored at -20°C decreased by only 5% after 9 days of storage. Bacteria in the preserved samples stored at 40°C and in the unpreserved frozen samples had at this time point reached 72% and 81% of the initial counts. After 16 days the bacterial numbers were reduced to 56-62% of the initial concentration in all treatments.

In conclusion, our results suggest rapid decay of bacteria and viruses in formaldehyde fixed water samples and the rates of decay were much higher than previously reported. One plausible explanation for this could be the higher tropical room temperature of transportation and storage. In addition, the higher organic matter content and biological activity of extracellular enzymes in highly eutrophic backwater samples may also be responsible for the faster rate of viral and bacterial decay in these samples. As previously suggested (Shibata et al. 2006), slides for enumeration should be prepared immediately or within 4 h of fixation and stored at -20 °C for accurate estimation of viral and bacterial counts.

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References

1. Fuhrman, J. A., Marine viruses and their biogeochemical and ecological effects. *Nature* (London) 1999, **399**, 541–548.
2. Bergh, O., Børsheim, K. Y., Bratbak, G. and Heldal, M., High abundance of viruses found in aquatic environments. *Nature* 1989, **340**, 467–468.
3. Proctor, L. M. and Fuhrman, J. A., Viral mortality of marine bacteria and cyanobacteria. *Nature* 1990, **343**, 60–62.
4. Suttle, C. A., Chan, A. M. and Cottrell, M. T., Infection of phytoplankton by viruses and reduction of primary productivity. *Nature* 1990, **347**, 467–469.
5. Wommack, K. E. and Colwell, R. R., Virioplankton in aquatic ecosystems. *Microbiol. Mol. Biol. Rev.*, 2000, **64**, 69–114.
6. Wilhelm, S. W. and Suttle, C. A., Viruses and nutrient cycles in the sea. *Bioscience*, 1999, **49**, 781–788.
7. Wilhelm, S. W. and Suttle, C. A., Viruses as regulators of nutrient cycles in aquatic environments, In *Microbial biosystems: new frontiers. Proceedings of the VIII International Symposium on Microbial Ecology* (ed. Bell, C. R., Brylinsky, M. and Johnson-Green, P.), Atlantic Canada Society for Microbial Ecology, Halifax, Nova Scotia, 2000, pp. 551–556.
8. Suttle, C. A., Ecological, evolutionary, and geochemical consequences of viral infection of cyanobacteria and eukaryotic algae, In *Viral Ecology*, (ed. Hurst, C. J.), Academic, New York, 2000, pp. 248–286.
9. Ananthanarayan, R. and Paniker, C. K. J., In *Textbook of Microbiology*, 7th ed. Hyderabad, Orient Longman, 2005.
10. Patel A, Noble R T, Steele J A, Schwalbach M S, Hewson I & Fuhrman J A, Virus and prokaryote enumeration from planktonic aquatic environments by epifluorescence microscopy with SYBR Green I. *Nat Protoc*, 2 (2007) 269-276.
11. Marie D, Brussaard C P D, Thyrhaug R, Bratbak G & Vaulot D, Enumeration of marine viruses in culture and natural samples by flow cytometry. *Appl Environ Microbiol*, 65 (1999) 45–52.
12. Brussaard C P D, Optimization of procedures for counting viruses by flow cytometry. *Appl. Environ Microbiol*, 70 (2004) 1506–1513.

13. Noble R T & Fuhrman J A, Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquat Microb Ecol*, 14 (1998) 113-118.
14. Chen F, Lu J R Binder B J, Li C & Hodso R E, Application of digital image analysis and flow cytometry to enumerate marine viruses stained with SYBR gold. *Appl Environ Microbiol*, 67 (2001) 539–545.
15. Hennes K P and Suttle C A, Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy. *Limnol Oceanogr* 40 (1995) 1050–1055.
16. Wen K, Ortmann A C & Suttle C A, Accurate estimation of viral abundance by epifluorescence microscopy. *Appl Environ Microbiol* 70 (2004) 3862–3867.
17. Gundersen K, Bratbak G, Heldal M, Factors influencing the loss of bacteria in preserved samples. *Mar Ecol Prog Serl*, 137 (1996) 305-310.
18. Shibata A, Goto Y, Saito H, Kikuchi T, Toda T & Taguchi S, Comparison of SYBR Green I and SYBR Gold stains for enumerating bacteria and viruses by epifluorescence microscopy. *Aquat Microb Ecol*, 43 (2006) 223-231.
19. Turley C & Hughes D J, Effect of storage on direct estimates of bacterial numbers of preserved seawater samples. *Deep Sea Res*, 39 (1992) 375-394.
20. Fahimi H D & Drochmans P, Purification of glutaraldehyde and its significance for preservation of acid phosphatase activity. *J Histochem Cytochem* 3 (1968) 199
21. Kolb-Bachofen V, Electron microscopic localization of acid phosphatase in *Tetrahymena pyriformis*: the influence of activities of lysosomal enzymes on fixation and structural preservation. *Cytobiologie* 15 (1977) 135-144.

Table 1. Percentage decrease in viral counts in waters samples preserved with various concentrations (1-6%)

formaldehyde

Days	% decrease in count \pm S.D Stored at 4°C						Stored at -20°C		
	1%	2%	3%	4%	5%	6%	W/o formaldehyde	1%	2%
2	32.4 \pm 0	28.3 \pm 1.5	27.6 \pm 0.6	19.6 \pm 2.2	24.7 \pm 2.4	21.6 \pm 1.4	ND	ND	ND
3	36.5 \pm 1.7	29.8 \pm 1.4	28.7 \pm 2.5	20.4 \pm 3.9	26.8 \pm 2.1	21.2 \pm 2.6	ND	ND	ND
4	37.6 \pm 1.3	30.8 \pm 1.6	29.5 \pm 0.2	22.5 \pm 4.1	28.5 \pm 1.6	31.4 \pm 1.8	ND	ND	ND
5	45.6 \pm 1.4	39.4 \pm 1.9	33.4 \pm 2.9	26.3 \pm 4.8	28.7 \pm 2.1	32.0 \pm 0.4	33.0 \pm 0.6	59.5 \pm 1.1	37.1 \pm 0.7
6	47.4 \pm 1.3	45.5 \pm 2.7	34.2 \pm 3.6	31.2 \pm 2.1	29.7 \pm 2.1	35.1 \pm 2.8	ND	ND	ND
7	47.9 \pm 1.2	47 \pm 0.9	41.7 \pm 1.9	34.1 \pm 5.2	35.3 \pm 2.8	39.1 \pm 1.6	ND	ND	ND
10	63.5 \pm 2.7	56.3 \pm 2.1	54.1 \pm 0.8	49.1 \pm 2.3	50.3 \pm 1.7	40.3 \pm 2	46.5 \pm 1.4	62.1 \pm 0.8	45.2 \pm 0.6
15	68.1 \pm 1.7	63 \pm 1.6	61.2 \pm 2.6	56.7 \pm 3	56.8 \pm 2.3	47.4 \pm 1.2	58.6 \pm 1.7	68.02 \pm 1.2	56.2 \pm 1.8
20	76.6 \pm 0.7	74.5 \pm 1.1	75.1 \pm 0.8	71.2 \pm 2.5	69.6 \pm 0.7	56.6 \pm 0.1	62.3 \pm 0.9	71.6 \pm 1.3	68.4 \pm 0.8
25	82.3 \pm 1.8	79.4 \pm 0.6	80 \pm 1.8	74.8 \pm 2.2	74.6 \pm 0.4	68.9 \pm 5.8	66.1 \pm 0.8	74.2 \pm 0.6	82.1 \pm 0.1
30	87.3 \pm .8	87 \pm 1	85.6 \pm 2.2	84.4 \pm 6.7	86.3 \pm 0.2	80.1 \pm 1.4	78.4 \pm 0.7	76.4 \pm 0.2	88.1 \pm 0.8
45	92.9 \pm 0.7	93 \pm 1.7	91 \pm 2.3	91.4 \pm 0.8	91.3 \pm 0.8	89.6 \pm 0.7	83.3 \pm 0.2	80.1 \pm 0.7	93.3 \pm 0.4

ND= Not Determined

Table 2. Percentage decrease in bacterial counts in waters samples preserved with various concentrations (1-6%) formaldehyde

Days	% decrease in count \pm S.D Stored at 4°C						Stored at -20°C		
	1%	2%	3%	4%	5%	6%	W/o formaldehyde	1%	2%
2	5.1 \pm 0.4	9.9 \pm 1	20.2 \pm 1.4	11.2 \pm 0.4	6.9 \pm 0.1	18.7 \pm 3.1	ND	ND	ND
3	12.1 \pm 0.6	12.1 \pm 0.4	38.6 \pm 0.1	26.5 \pm 0.2	31.9 \pm 0.7	43.8 \pm 0.7	ND	ND	ND
4	31.5 \pm 0.9	15 \pm 0.7	44.4 \pm 1.0	33.4 \pm 1.0	38 \pm 0.1	44.4 \pm 1.2	ND	ND	ND
5	40.7 \pm 0.4	21.1 \pm 0.4	51.4 \pm 1.1	50.1 \pm 0.1	47 \pm 0.1	44.9 \pm 0.9	36.0 \pm 0.1	42.1 \pm 0.7	30.1 \pm 0.5
6	52.3 \pm 0.1	22 \pm 0.3	54.6 \pm 0.8	52.8 \pm 0.4	50.4 \pm 0.8	47.7 \pm 0.1	ND	ND	ND
7	53.3 \pm 0.6	23.4 \pm 0.3	60.0 \pm 0.3	55.1 \pm 1.5	51 \pm 1.4	48.5 \pm 0.5	ND	ND	ND
10	54.6 \pm 0.7	28.7 \pm 0.3	60.4 \pm 0.1	57.9 \pm 0.4	53.2 \pm 1.1	51.1 \pm 2.0	42.3 \pm 0.5	51.2 \pm 0.4	38.2 \pm 0.7
15	55.7 \pm 0.4	36.1 \pm 0.4	60.8 \pm 0.1	61.1 \pm 0.5	56.3 \pm 0.1	51.8 \pm 1.3	55.4 \pm 0.8	58.6 \pm 0.8	44.4 \pm 0.2
20	59.3 \pm 0.7	54.6 \pm 0.7	66.6 \pm 1.2	65.1 \pm 0.4	58.2 \pm 1.1	57.8 \pm 1.2	62.7 \pm 1.7	63.4 \pm 0.2	52.3 \pm 0.5
25	65 \pm 0.7	67.3 \pm 0.8	68.9 \pm 0.2	65.4 \pm 0.1	68.2 \pm 0.9	62.35 \pm 0.1	76.4 \pm 0.2	71.8 \pm 1.2	64.8 \pm 0.9
30	75.8 \pm 0.5	71 \pm 0.8	78.7 \pm 0.9	77.6 \pm 1.3	79.5 \pm 0.7	69 \pm 0.4	80.5 \pm 1.3	78.2 \pm 1.3	73.7 \pm 0.8
45	85.3 \pm 0.3	89.2 \pm 1.0	89.5 \pm 0.5	88.1 \pm 0.6	87 \pm 0.2	89 \pm 0.1	87.1 \pm 0.8	82.1 \pm 0.4	87.2 \pm 0.3

ND = Not Determined

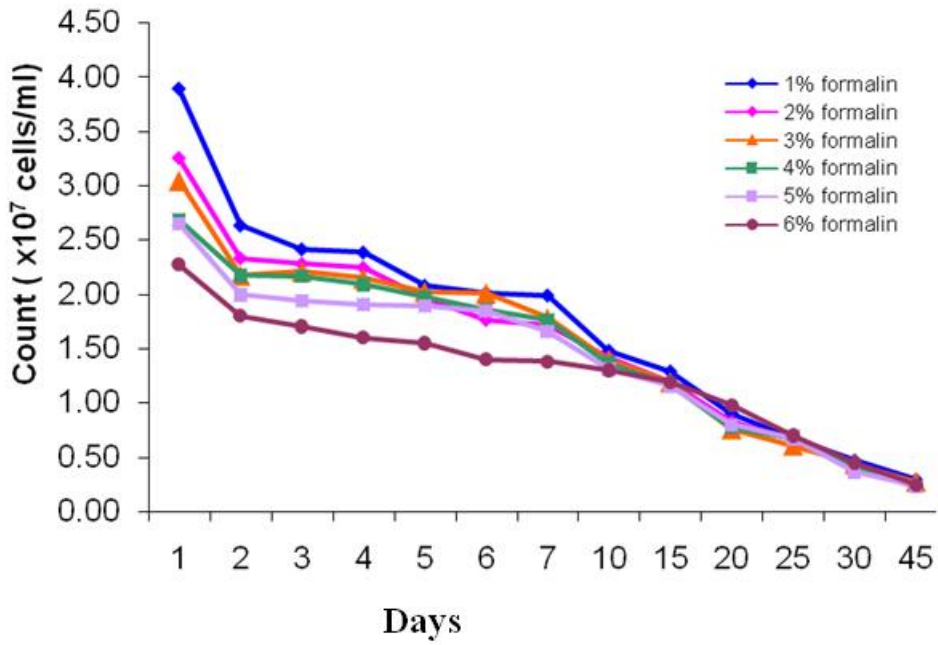


Figure 1. Changes in viral counts of water samples preserved with various concentrations (1-6%) of formaldehyde

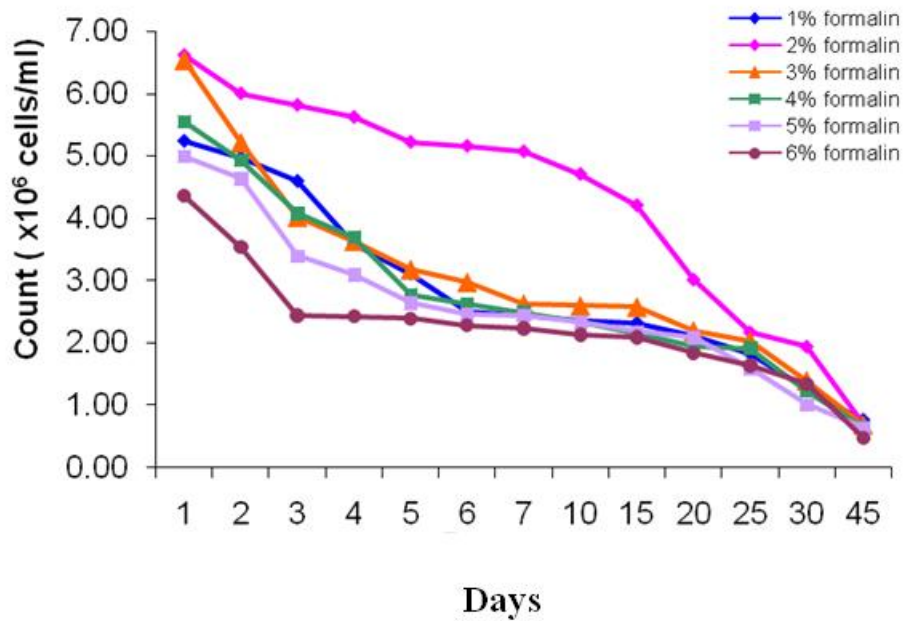


Figure 2. Changes in total bacteria counts of water samples preserved with various concentrations (1-6%) of formaldehyde