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Survival of *Campylobacter jejuni* in mineral bottled water according to difference in mineral content and presence of the heterotrophic flora: application of the Weibull model

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Abstract

The aim of the study was to examine the hypothesis emitted by Evans et al. (2003) that mineral bottled water accidentally contaminated by *Campylobacter jejuni* would represent a risk factor for C*ampylobacter* infection.

Culturability of *C. jejuni* cells inoculated in low and high mineral bottled water during storage at 4°C in the dark was performed by surface plating and modelled using the Weibull model. The loss of *C. jejuni* culturability observed in all conditions tested was shown to be dependent from strain, preculture condition and water composition.

Following inoculation of *C. jejuni*, the rapid loss of culturability was not correlated to complete cell death as the passage into embryonated eggs enabled recovery of cells from the Viable But Non Culturable state.

In conclusion, the sanitary risk associated to contaminated bottled water can not be excluded although it is presumably low. Culture conditions, strain and water type must be taken into account in the evaluation of the risk factor as they influence significantly *Campylobacter* survival in water.

Key words: *Campylobacter jejuni*, culturability, VBNC, bottled water, risk factor

Introduction

Campylobacter is generally recognized as the most common cause of bacterial gastroenteritis world wide. However the etiology of this pathogen remains partly understood. Indeed recognized outbreaks are rare and are usually caused by poultry, milk or contaminated water (Pebody et al., 1997; Frost et al., 2002). In most cases, symptoms associated with *Campylobacter* are common (diarrhea followed by rapid recovery). Therefore the identification of the pathogen and the contaminated food product is not often requested by medical services and it makes the elucidation of risk factors difficult for sporadic *Campylobacter* infections.

Campylobacter is a waterborne pathogen, which can survive for extended periods in natural aquifers after deposition by animal hosts, in the form of Viable But Non Culturable cells (Rollins and Colwell, 1986). Water is thought to be one of the main transmission routes of Campylobacteriosis (Koenraad et al., 1997). Outbreaks of *Campylobacter jejuni* due to consumption of tap water have recently occurred in Wales (Richardson et al., 2007). Drinking bottled water has recently been identified as a possible risk factor for *Campylobacter* infection (Gillespie et al., 2002). Evans et al. 2003 examined this hypothesis and conducted a retrospective cohort study by mailing a questionnaire to the *Campylobacter* case-patients to investigate the cause of sporadic *Campylobacter* infection (Evans et al., 2003). Their study identified five independent risk factors for *Campylobacter* infection including three risk factors attributed to food and the other two to contact with animals. Among these risk factors, eating chicken was the most important, explaining 31% of *Campylobacter* infections in the model, then came eating salad vegetables for 21% and then drinking bottled water for 12%. Storage of bottled water during several months before consumption might favour proliferation and / or survival of microorganisms accidentally present in water and sustain the hypothesis

of campylobacteriosis attributed to consumption of bottled water. Survival of *C. jejuni* in simple water systems has been the subject of several studies (Cools et al., 2003). However no experimental study has been conducted yet to examine the potentiality of bottled water to represent a risk factor for *Campylobacter* infections as it is sustained by the statistical cohort study led by Evans et al. (2003). Indeed no decontamination treatment is performed before bottling of natural mineral water and bottled water may be stored for months before sale enabling contaminants to proliferate if present. Therefore the ability of pathogens to survive in bottled mineral water may represent a public health concern we proposed to examine in this paper. In most studies, filtered and sterile water were used as microcosms to determine *Campylobacter* survival in water. Moreover survival curves are rarely modelled. We chose to inoculate bottled water without previous filtration in order to examine the real potential of persistence of *C. jejuni* in bottled water resulting from an accidental contamination before bottling. The potential antagonistic or synergistic effect of water heterotrophic flora was also investigated.

In this purpose, two bottled mineral waters chosen according to their difference in mineral content were artificially contaminated with two strains of *C. jejuni*, a wild (Bof) and a reference strain (NCTC 11168). *Campylobacter* survival in mineral water and possible recovery from the Viable But Non Culturable state (VBNC) were examined during storage of 48 days respectively by colony counting and passage into embryonated eggs, in conditions favouring longest cultivability (obscurity and 4°C). Influence of the heterotrophic flora of water on *C. jejuni* survival was also evaluated.

Because survival curves may adopt different shapes in function of the nature of the stress and its intensity, the strain and its physiological state, we did not use the frequently simple log-linear inactivation model. The cumulative form of Weibull frequency distribution model was used to describe *Campylobacter* survival kinetics in water as it is suitable for quantifying

bacterial survival for both log and non-log linear survival curves. The Weibull model has largely been used to describe bacterial inactivation by thermal and nonthermal processes as stress resistance of a microbial population often follows a Weibull distribution (Cunhan et al., 1998; Peleg and Cole, 1998; Fernandez et al., 1999; van Boekel, 2002; Corradini and Peleg, 2003; Virto et al., 2005; Hajmeer et al., 2006). We used a logarithm decimal form of Weibull defined as follows (Mafart et al., 2002; van Boekel, 2002):

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^p \qquad \text{Eq1}$$

In this model δ corresponds to the first reduction time that leads to a 10-fold reduction of the surviving population. Parameter p characterises the shape of the curves: concave curves $p < 1$, convex curves $p > 1$ and linear curves p equal 1, in this case δ parameter can be assimilated to the traditional D value of thermal resistance curves. This equation was used by different authors (Mafart et al., 2002; Virto et al., 2005; Carlin et al., 2006; Buzrul, 2007) and was implemented in this work as primary model. The shape parameter p variation on the goodness of fit of models was shown by Couvert et al. (2005) to have slight influence so that a single p-value can be used for a given microbial population (same physiological state, same preculture) (Couvert et al., 2005). A single overall estimation of p-value per strain was determined, regardless of environmental conditions (water type) and recovery (medium used for colony counting), as it seemed to be enough for bacterial food predictive modelling and canning process calculation (Mafart et al., 2002).

Material and methods

Microorganisms : *Campylobacter jejuni* NCTC 11168 (reference strain) and Bof (wild type isolated from human enteritis) were used for the study. Laboratory stocks of cells were stored frozen in cryotubes at –70°C. Cells were revived by plating on Karmali medium (Merck). Karmali plates were incubated in a controlled, gaseous environment (Binder CO_2 incubator; 42°C, 10% CO_2, 5% O_2, 85% N_2) during 48 h. Cells were subcultured twice on Columbia Blood Agar (CBA, Merck) containing 5% horse blood for 24 h in the same atmospheric and temperature conditions.

Inoculum preparation

C. jejuni survival in bottled water was studied as a function of the type of preculture – broth or agar – used for inoculation.

Preculture in broth

Preculture in broth consisted in harvesting cells from a 24-h-old CBA plate with a sterile loop and resuspended them into 100 ml Mueller Hinton Broth (Biokar Diagnostics). Cells were incubated at 42°C for 24 h in the CO_2 incubator (10% CO_2, 5% O_2, 85% N_2) and harvested by centrifugation (8 000 g, 15 min). The supernatant was discarded and the pellet washed twice in 50 ml mineral water (same conditions of centrifugation).

Preculture on agar

Preculture on agar consisted in subculturing cells from a 24-h-old CBA plate on another fresh one. Cells were incubated at 42°C for 24 h in the CO_2 incubator (10% CO_2, 5% O_2, 85% N_2) and harvested with a sterile loop resuspended in 50 ml mineral water and washed twice (8 000 g, 15 min).

Inoculation of *Campylobacter jejuni* in mineral bottled water

Bottles containing low or high mineral water (see composition in Table 1) were inoculated with 10^7 to 10^9 *C. jejuni* cells per ml (NCTC 11168 or Bof strain) from broth or agar

preculture. Bottles were stored in the dark at 4°C during 48 days. Experiments were performed in twice.

Cell survival in mineral bottled water

Survival of *C. jejuni* was determined by plate counts on CBA (non selective) and Karmali (selective) medium incubated at 42°C in jars conditioned with microaerobic atmosphere (5% O_2 and 10% CO_2) for 48 h.

Recovery in embryonated eggs of *C. jejuni* Viable But Non Culturable cells

After 48 days of storage, in water containing less than 1 CFU (Colony Forming Unit) per 2 ml as determined by the plate count technique, filtration of the whole bottle of mineral water and enrichment in Preston broth according to the NF-ISO 10272 norm were performed in order to enhance the detection threshold and detect remaining culturable cells (Talibart et al., 2000). One ml of water in which no culturable cell was detected, was inoculated into embryonated eggs in order to recover *C. jejuni* VBNC cells potentially present and not detected by traditional cultural techniques.

Negative control consisted in inoculating 1 ml of sterile distilled water in an embryonated egg. Two positive controls were used in which 1.5 ml of mineral water containing NCTC 11168 or Bof cells harvested from a 24-h-old CBA plate were inoculated in embryonated eggs. Eggs were incubated at 37°C during 7 days in microaerophilic conditions. Eggs were then broken and yolk was ensemenced on CBA and incubated at 37°C in microaerobic conditions. Four eggs were used by condition tested.

Heterotrophic plate counts

Heterotrophic plate counts were determined every two or three days during storage at 4°C of bottled mineral water inoculated or not with *C. jejuni* by surface plating on R2A medium (Difco) and incubation at 20°C for 5 days.

Fitting parameters and confidence interval determination

Survival curves of *Campylobacter* were fitted by the following decimal logarithm form of Weibull model (Eq 1). In our experiments, a single p-value was calculated and used to describe the survival of a given strain after a given preculture in order to evaluate the influence of water mineral content on *Campylobacter* survival using two different media for viable counts. The parameter values and their associated confidence interval were fitted by using a non-linear module (bnlinfitQ and bnlparciQ Matlab 6.1, The Mathworks) (Couvert et al., 2005). To appreciate the accuracy on the non-linear models used in this study F test and associated probability p were carried out.

Experimental design

Four bacterial populations were obtained resulting from the association of a strain type, Bof or NCTC 11168, and a preculture medium, agar or broth. For each of these 4 populations a single p value was estimated and bacterial survival kinetics were performed in low mineral water (LMW) and high mineral water (HMW) using two enumeration media: CBA and Karmali medium. For each condition δ parameters were estimated.

Results

Influence of storage in water on *Campylobacter jejuni* survival

Fig. 1 describes survival of *C. jejuni* Bof and NCTC 11168 strains during storage in low and high mineral water at 4°C. The Weibull model used to model cell survival during storage provided a good fit, enabling the study of *Campylobacter* survival in water through the variation of p and δ fit parameters (Table 2). Results showed that the p parameter associated to the shape of survival curves was dependent from the type of preculture as it was supposed by Couvert et al. (2005). Concave upward survival curves were observed for all kinetics and attested by a p value superior to one. However, the p value was shown to be higher for

survival curves obtained from cells precultured in broth, than from cells precultured on agar. Similar p values were found for both strains for a given preculture medium.

Results showed that the initial viable population of *C. jejuni* decreased in low and high mineral water during storage in the dark at 4°C for both strains (Fig. 1). Although similar tendencies could be observed with both enumeration media, enumeration on Karmali medium resulted in significantly lower viable counts and δ values ($P < 0.05$) than on CBA (Fig. 1). Viable cells of *C. jejuni* could be detected in water by CBA plate counts until 28 to more than 48 days of storage at 4°C in the dark depending on experimental conditions (mineral content of water and strain type). Statistical analysis of delta values showed that there are statistically significant mineral content, strain and preculture effects ($P< 0.05$). Survival in high mineral water was shown to be higher than in low mineral water. Bof strain was found to be more resistant to storage at 4°C in the dark than the reference strain. Finally, strains obtained from agar preculture were shown to be more sensitive to storage in water than those obtained from broth preculture.

Recovery of VBNC cells in embryonated eggs

After 48 days, in water containing no culturable cells after filtration and enrichment in Preston broth, recovery of VBNC cells was assayed. This could be performed only for the NCTC 11168 strain as the Bof strain remained culturable after 48 days. Table 3 shows the recovery of culturability of VBNC *C. jejuni* cells after passage in embryonated eggs and incubation during 7 days. In high mineral water culturability of VBNC *C jejuni* NCTC 11168 strain is recovered whatever the preculture type. In low mineral water, for both preculture types, recovery is observed in half of the cases. It could be noted that in low mineral water no colony was observed on CBA media after 32 days whereas loss of culturability on CBA occurred after more than 40 days in high mineral water (Fig 1).

Influence of heterotrophic flora on *C. jejuni* survival in water

Heterotrophic flora was determined in water inoculated or not with *C. jejuni*. Inoculation of *C. jejuni* induced an increase of the heterotrophic flora, thereafter; it remained relatively constant throughout the storage at 4°C in both waters (Fig. 2).

Discussion

Influence of storage in water on *Campylobacter jejuni* survival

Survival of *C. jejuni* was studied in low and high mineral bottled water in the dark at 4°C. Because survival curves may adopt different shapes in function of the nature of the stress and its intensity, the strain and its physiological state, we did not use the frequently simple log-linear inactivation model but the Weibull model. Results showed that the initial culturable population of *C. jejuni* decreased in low and high mineral water during storage in the dark at 4°C for both strains. The decline of *C. jejuni* in water environment has been dealt in numerous papers (Cappelier et al., 1997; Buswell et al., 1998; Thomas et al., 1999; Tatchou-Nyamsi-Konig et al., 2007). This result is not far different from those obtained by Thomas et al. (1999) and Tatchou-Nyamsi-Konig et al. (2007) respectively in sterile river water and in filtered mineral water. Tatchou-Nyamsi-Konig et al. showed that survival of thermophilic *Campylobacter* was better at 4°C than at 25°C, which confirms the choice we have made to maintain *Campylobacter jejuni* strains at a most favourable temperature for survival. However NCTC 11168 *C. jejuni* strain survived longer in the experiments of Thomas et al. with sterile river water (more than 60 days) than in ours conducted in low and high mineral bottled water. Cools et al. (2003) indicated that survival duration of *C. jejuni* in water could vary from 2 to 4 weeks (Chan et al., 2001) to more than 4 months (Rollins and Colwell, 1986; Cools et al., 2003). These differences were supposed to be attributed to strain variations, differences in experimental conditions or in the methodology of culturability assessment. A strain effect was indeed observed in our experiments. The Bof strain was found to be significantly more

resistant to storage at 4°C in the dark than the NCTC 11168 reference strain. This result is consistent with those obtained by Cools et al. (2003) who found that the origin of the strain was a determining factor for the survival of *C. jejuni* in drinking water at 4°C.

Selective and non-selective media were used for viable counts. Results showed that the loss of culturability occurred sooner by enumeration on Karmali (Fig. 1). This was already observed by Cools et al. (2003) and was attributed to the better recovery of starvation-stressed bacteria in non-selective media. Enumeration on selective medium might underestimate cell viability as it is often observed when microorganisms are stressed.

We observed that the survival of *C. jejuni* was higher in high mineral water than in low mineral water. Mineral content of water seems to affect significantly *C. jejuni* survival at low temperature.

Finally, strains obtained from agar preculture were shown to be more sensitive to storage in water than those obtained from broth preculture. History of the culture conditions of *Campylobacter* predating to accidental contamination of water might influence significantly their subsequent survival. This was previously observed by Duffy and Dykes (2006) for growth temperature.

Recovery of VBNC cells in embryonated eggs

Several studies aiming the behaviour of bacteria submitted to adverse environmental conditions show that some bacteria can lose their capability to multiply and consequently to form colonies on nutrient agar. However they remain viable as attested by the persistence of metabolic activity demonstrated by the Direct Viable Count method and the measurement of respiratory activity (Cappelier et al., 1997; Cappelier and Federighi, 1998; Federighi et al., 1999; Tholozan et al., 1999). This physiological state was called Viable But Non Culturable (VBNC). We observed that VBNC NCTC 11168 *C. jejuni* cells could be recovered through passage in embryonated eggs. It means that NCTC 11168 cells might lose rapidly their ability

to grow on agar plates and become VBNC. Hence, they are rapidly no more detectable by traditional plate count technique but some of them are nevertheless present in the state of VBNC cells. A better recovery of VBNC cells in high mineral water than in low mineral water (Table 3), suggesting that in addition to favour better *C. jejuni* survival, high mineral water enables the generation of VBNC cells. One-hundred percent *C. jejuni* VBNC recovery could be obtained by Cappelier et al. by the technique using embryonated eggs (Cappelier et al., 1997). The low recovery obtained in experiments performed in low mineral water suggests that few VBNC cells are present after a 48-day storage in low mineral water.

Influence of heterotrophic flora on *C. jejuni* survival

Heterotrophic plate counts (HPC) also termed as autochthonous flora are commonly used to assess the general microbiology of water. The most commonly isolated genera of these microorganisms that use organic compounds for most or all of their carbon requirements in drinking water systems are associated to *Aeromonas, Acinetobacter, Aureobacterium, Bacillus, Chryseobacterium, Corynebacterium, Klebsiella, Moraxella, Pseudomonas, Staphylococcus, Tsukmurella* and *Vibrio* (Pavlov et al., 2004). Under this broad definition, primary and secondary bacterial pathogens are included, as are coliforms (*Escherichia, Klebsiella, Enterobacter, Citrobacter, Serratia*). These bacteria may come from the water environment or from other diverse pollutant sources. Many studies seemed to reveal that these bacteria are mostly harmless. But recently, potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water could be observed (Pavlov et al., 2004). Most papers dealing with heterotrophic flora wonder about their possible association with health risk examining their cytotoxicity and invasiveness capabilities (Allen et al., 2004). However, the indirect influence of HPC on the establishment and survival of other pathogens has not extensively been studied. Indeed, lysis of

autochthonous flora resulting from cell death might provide nutrients for pathogens, hence enabling their survival in nutrient-starved environments such as bottled water. In the same way, cell death of pathogens might induce an increase in HPC in that nutrients are released in water. An increase in HPC was observed following introduction of *C. jejuni* in bottled water (Fig. 2). Analysis of Fig. 1 showed that the population of *C. jejuni* was not increased during storage in water but at the opposite that a decrease in culturable cells occurred since the first days of storage. The presence of HPC did not favour the establishment and maintenance of culturability of *C. jejuni*. The fact that *C. jejuni* did not survive either in sterile water (data not shown) demonstrated that *C. jejuni* mortality in bottled water was not due to competition or any antagonistic activity of HPC cells although this latter characteristic has been them assigned (Tamagnini and Gonzalez, 1997; Vachee et al., 1997). However Tatchou-Nyamsi-Konig et al. did not observe any effect of the autochthonous flora on *C. jejuni* survival in natural mineral water either (Tatchou-Nyamsi-Konig et al., 2007). On the other hand, the increase in HPC resulting from the introduction of *C. jejuni* in bottled water might be due to their growth over *C. jejuni* dead cells.

Conclusions

(1) The Weibull model was successfully used to model *C. jejuni* survival in water.

(2) We showed that in conditions favouring *C. jejuni* survival in water (low temperature, obscurity, no agitation), culturability of *C. jejuni* decreased progressively in mineral bottled water. The bacteria remain detectable by traditional techniques until 28 to more than 48 days depending on preculture, water and strain type.

(3) The strain type, the mineral content of water effect and the nature of the preculture were shown to influence significantly the maximum duration of *C. jejuni* cultivability.

Longer survival was found for the Bof strain, in high mineral water and after a preculture in broth.

(4) We have proved that some *Campylobacter* are viable but non-cultivable after storage in bottled water at 4°C and able to resuscitate after a passage into 7-day fertilised eggs. Storage in mineral bottled water constitutes obviously stress conditions resulting in loss of culturability and generation of VBNC cells.

(5) HPC was shown to have neither antagonistic nor favourable influence on *C. jejuni* survival in low and high mineral water at 4°C.

(6) This study aimed to examine the hypothesis emitted by Evans et al. about the potential risk factor represented by bottled water contaminated with *C. jejuni*. The culturability loss and the low level of recovery of VBNC cells suggest that consumption of bottled water would unlikely represent a risk for human health. However, caution has to be taken as culture conditions, strain and water type influence significantly *Campylobacter* survival.

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Illustrations and Tables

Figure 1. Fitted survival curves of NCTC 11168 (a, c) and Bof (b, d) strains of *Campylobacter jejuni* determined by plate counts on Karmali media (open symbols, dashed lines) and on CBA (closed symbols, plain lines) during storage in high (a, b) and low (c, d) mineral drinking water at 4°C in the dark after preculture on agar (▲, △) and in broth (●, ○).

Figure 2. Evolution of heterotrophic flora of water during storage at 4°C resulting from inoculation of NCTC 11168 (▲; △) and Bof (●; ○) *C. jejuni* strains in low (a) and high (b) mineral water. (+)represents the enumeration obtained without inoculation of any strain. Open symbols refer to agar preculture and closed symbols to broth precultures. Heterotrophic flora was determined by enumeration on surface-plated R2A medium after 5 days at 20°C.

Table 1. Composition of bottled mineral waters ($mg.l^{-1}$)

Table 2. Fit parameters p and δ obtained according to the Weibull model for *C. jejuni* survival in Low Mineral Water (LMW) and High Mineral Water (HMW)

Table 3. Recovery in embryonated eggs of VBNC cells of NCTC 11168 *C. jejuni* potentially present in water containing less than 0.5 $CFU.ml^{-1}$. 1 ml of mineral water was inoculated and incubated in embryonated eggs during 24h and allowed to recover at 42°C during 7 days. Results express the rate of embryonated eggs in which *C. jejuni* could be recovered out of 4.

Table 1
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Table 1

	Low mineral water	High mineral water
Calcium	**12**	**84.5 (x 7)**
Natrium	13	7
Magnesium	**9**	**27 (x 3)**
Khalium	6.5	1.1
Bicarbonate	**79.3**	**361.1 (x5)**
Chloride	12.5	6
Sulfate	7	12
Nitrate	6	3.8
Silica	36	15
Fluoride	0.14	< 0.1

Table 2

Viable counts enumeration medium		Strain / preculture type							
		11168 / agar		11168 / broth		Bof / agar		Bof / broth	
	p	value	CI	value	CI	value	CI	value	CI
		1.95	0.29	2.22	0.34	1.80	0.20	3.00	039
Karmali medium	δ_{LMW} (days)	9.05	1.30	12.02	1.59	1.59	1.40	20.83	1.69
	δ_{HMW} (days)	9.30	1.43	17.78	2.31	2.31	2.12	25.80	2.00
CBA medium	δ_{LMW} (days)	11.48	1.56	13.28	1.71	1.71	1.89	26.57	2.01
	δ_{HMW} (days)	10.50	1.57	19.64	2.39	2.39	2.26	28.61	2.10

CI: Confidence Interval for $P < 0.05$

Table 3
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Table 3

	Low mineral water	High mineral water
agar preculture	2/4	4/4
broth preculture	2/4	4/4

Figure 1

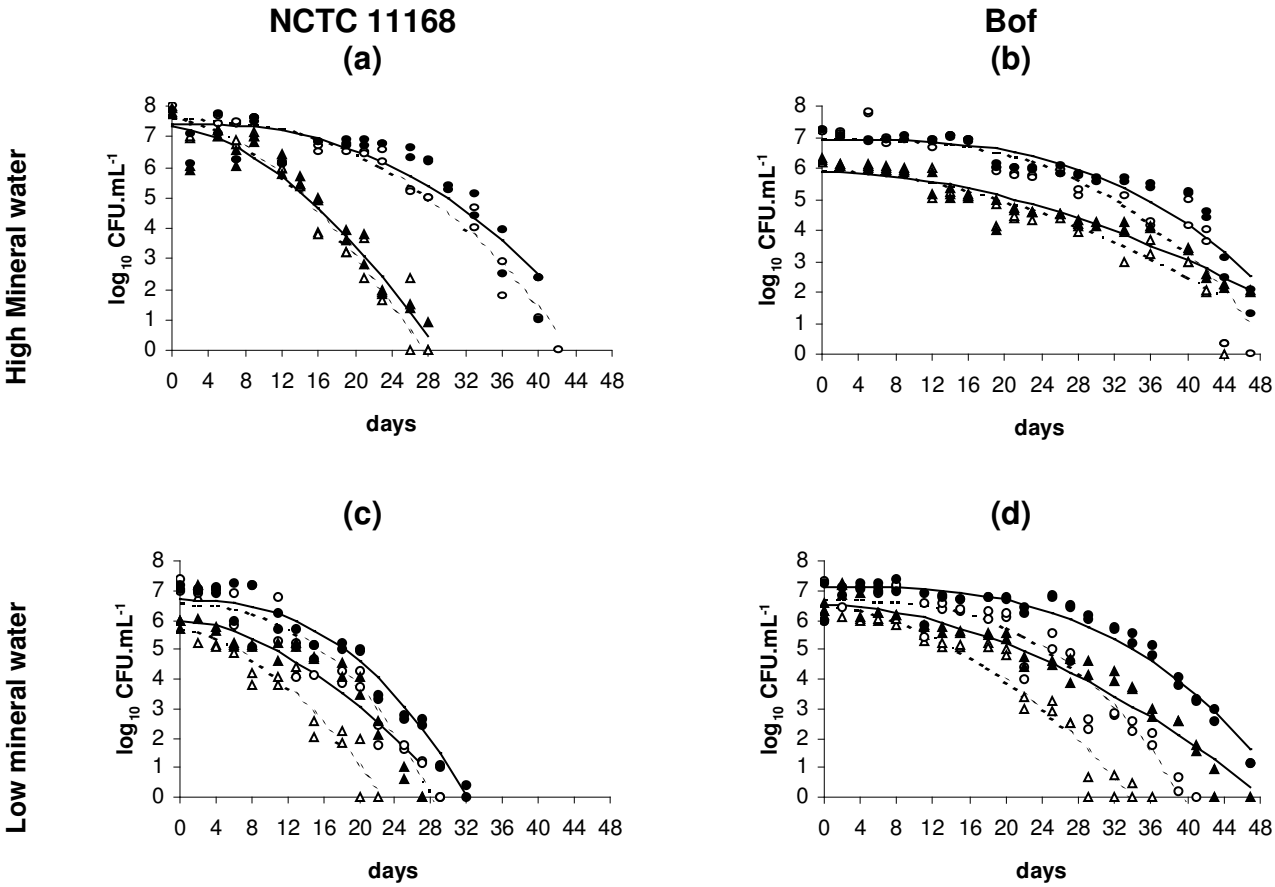
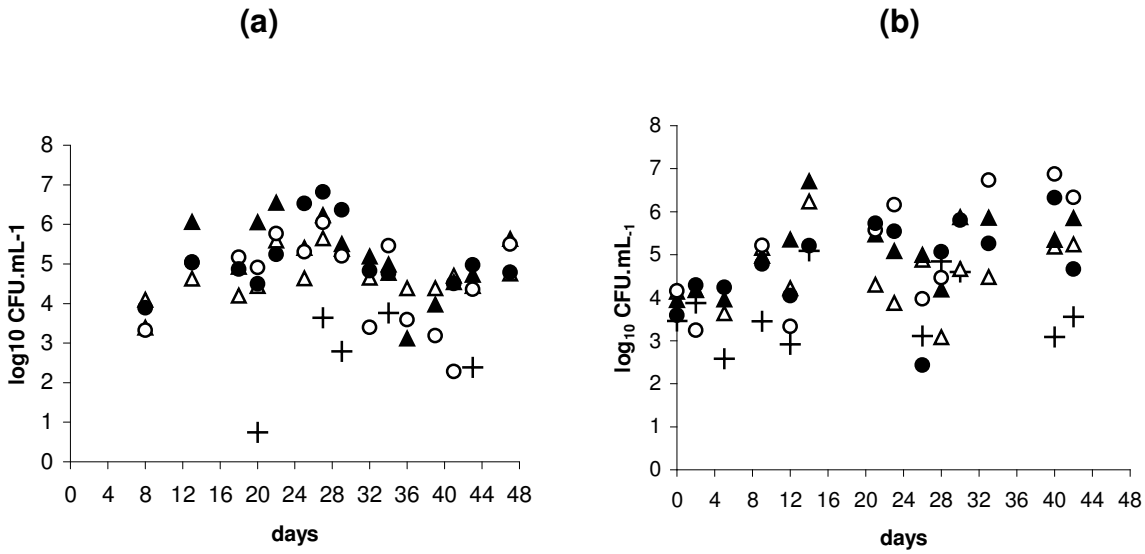

NCTC 11168
(a)
Bof
(b)
High Mineral water
Low mineral water
(c)
(d)
log10 CFU.mL-1
days

Figure 2


(a)
(b)
log10 CFU.mL-1
log10 CFU.mL-1
days
days
0 4 8 12 16 20 24 28 32 36 40 44 48
0 1 2 3 4 5 6 7 8