Microbial Quality and Nutritional Aspects of Norwegian Brand Waters.

Eli Otterholt[1] and Colin Charnock[1*]

[1]Department of Health Sciences, Oslo University College

*Corresponding author. Mailing address: Colin Charnock, Department of Health Sciences, Pilestredet 46, 0167 Oslo.

Tel.: +47-22-452348

Fax.: +47-22-452335

E-mail: colin.charnock@hf.hio.no

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Abstract

The microbiological quality of the five leading brands of Norwegian bottled still waters was investigated. All brands were free for the enteric indicator organisms and named pathogens whose absence is demanded in current quality directives. The relatively nutrient-poor agar R_2A revealed large heterogeneous bacterial populations which grew slowly, or not at all, on clinical media specified for use in substrate-utilization approaches to identification. The main approach used for identification was cultivation of microbes on R_2A, followed by amplication and partial sequencing of 16S rDNA genes. The identity of the heterotrophic plate count of the brands differed significantly to that found in many other similar studies with respect to the dominating species. The bacterial flora was dominated by beta- and alphaproteobacteria most of which were psychrotolerant. Several brands contained *Sphingomonas* and large populations of *Methylobacterium* species which have been associated with a variety of opportunistic infections of immunocompromised hosts. Analysis of the isolated strains nutritional capabilities using the Biolog GN2® system, gave in most instances low positive scores, and strain identifications using the system were generally inconclusive. Measures of assimilable organic carbon in the water revealed that some brands contained levels higher than those which have been associated with biological stability and restricted or no growth of heterotrophs in distribution systems. The relationship between assimilable organic carbon and R_2A plate counts was significant and moderately positive for bottled waters. Assimilable organic carbon correlated strongly with the survival time of *Escherichia coli* when introduced into bottles as a contaminant. Those brands having high values (~ 100 µg/L) supported protracted survival, but not growth of *E. coli*, whereas *E. coli* quickly became nonculturable in brands with low values. Thus measures of assimilable organic carbon may have a particular value in predicting the survival of this and nutritionally

similar species of hygienic relevance. Only small numbers of fungi were found. However, one isolate (*Aureobasidium pullans*) has been associated with infections of humans.

1. Introduction

Effective marketing and a general scepticism to the quality of tap water has over the past decade resulted in a marked increase in the consumption of bottled/dispenser waters (Liguori et al., 2010). In Norway, an outbreak of *Giardia* disease in Bergen in 2004, detection of this parasite in Oslo's tap water in 2007 leading to a boil water recommendation, and concerns about the poor quality of the distribution net have raised public concerns about tap water quality. There is a general perception that bottled water is purer and safer for human consumption, and targeted groups for marketing have included infants and immuno-compromised individuals (Papapetropoulou, 1998; Bharath et al., 2003). Current Norwegian legislation governing the quality of bottled waters define natural mineral water (NMW) and spring water (SW) and provide guidelines for the treatment, bottling and microbiological quality of these (Helse- og omsorgsdepartementet, 2004). The Norwegian document adopts the general definitions and guidelines for microbiological quality given in the current European legislature (Directives 80/777/EEC and 96/70/EC of the European Parliamnet and of the Council). The proven absence of coliforms, *Escherichia coli*, *Enterococcus* sp. and anaerobic spore-forming sulphite-reducing bacteria is demanded. Furthermore, the heterotrophic plate count (HPC) should reflect a source water free from contamination. As neither treatment nor bottling or materials shall otherwise change the natural composition of the water, large bacterial populations usually develop from small initial populations shortly after production (Loy et al., 2005 and references therein). However, few characterizations of the natural heterotrophic plate count microbes (including fungi) exist and to our knowledge there are no such published analyses of Norwegian bottled waters. The HPC as a quality control parameter has several weaknesses: only a small fraction of the total viable microbial population grow on artificial laboratory media (Watkins and Xiangrong, 1997). Furthermore, it is now generally accepted that in the absence of faecal contamination there is no direct

relationship between HPC values in ingested water and human health effects in the population at large (Expert Meeting Group, 2003). However, HPC continues to figure in water regulations and guidelines in most countries as an indicator of the general microbiological quality and, as such, still requires attention. The test is inexpensive, quick and reliable and the HPC composition can indicate changes in water quality which should be evaluated as a trigger for further investigation (Expert Meeting Group, 2003). Furthermore, specific strains which might be a part of the HPC microbiota can cause infection of immunocompromised people and there is at present insufficient epidemiological data to evaluate the relevance of HPC microbes for these groups (Expert Meeting group, 2003; Pavlov et al., 2004). The highest HPC values for water are obtained with low nutrient media and long incubation times. Massa et al., (1998) showed that R_2A medium gave plate counts exceeding those on plate count agar used in other studies of bottled waters (eg Pavlov et al., 2004) by more than 300%. Furthermore, *Flavobacterium* spp. and *Arthrobacter* spp. were only recovered on R_2A which the authors subsequently recommend for analysis of NMW. While there have been many studies of the bacterial species found in water, the identification of bacterial isolates from the environment has previously been impaired by a poor database. It is highly probable that many of the identifications reported in the literature over the years are incorrect. Many were made employing clinical systems for which the database was not appropriate for environmental strains. Molecular methods have changed our views of the "species," and we should at least question many of the bacterial identifications in the literature (Expert Meeting Group, 2003).

The reasons for the development of a HPC in waters after bottling are still a matter of debate. The influence of materials, the relative contributions of attached (eg, biofilms) to unattached microbes, and of regrowth (from small initial populations) as opposed to resuscitation of existing microbes show the complexity of the issue (see discussion in Expert Meeting Group, 2003). An aspect which so far has received little attention, is a possible

correlation between the development of a HPC in bottles and measures of the fraction of dissolved organic carbon which can be used in the production of bacterial biomass. The assimilable organic carbon (AOC) analysis (Van der Kooij et al., 1999) involves the addition of 1-2 heterotrophic stock strains to pasteurized water samples. The combined metabolic capabilities and carbon assimilatory capacity of the strains should, ideally, result in a growth response which gives a good indication of the amount of carbon that can be converted to biomass by indigenous species. The units are carbon equivalents derived from growth of the assay strains on model carbon sources, usually acetate and oxalate. It has been observed for groundwater distribution systems that when AOC values approached 10-15 μg C/L little or no regrowth of heterotrophic bacteria occured and the HPC remained low at < 100 CFU/mL (Van der Kooij et al., 1999 and references therein). It has also been reported that waters with AOC levels of > 100 μg C/L gave 82% more positive coliform samples (LeChevallier et al., 1999). AOC measurements have not been previously used in the analysis of bottled waters, but bacterial growth during storage might by comparison to tap water be related to high AOC values. If so this might provide the analyst with a tool for predicting the development of a HPC population and more importantly the survival of enteric pathogens.

The numbers, types and resistance profiles of fungi causing infections have increased dramatically in recent years. There are increasing numbers of severe fungal infections, particularly aming immunocompromised people by commensal or fully saprophytic species (Enoch et al., 2006). The role of drinking water in the dissemination of fungal pathogens is still a matter of debate (Expert Meeting group, 2003). There is thus a need to evaluate the fungal fraction of drinking waters and to monitor any changes in its status. The explicit requirement for the absence of pathogenic organisms in all bottled water types (Helse- og omsorgsdepartementet, 2004) would naturally encompass fungal pathogens. However, fungi

as a group are not mentioned in the Norwegian drinking water directives. Methods for detection are not provided and to what extent fungi are tested for is not known.

The main focus of the current study is to investigate the microbiological quality of the leading brands of Norwegian bottled waters. It seeks to characterize the bacterial and fungal HPC using particularly genotype-based methodologies. Factors, particularly nutritional capabilities of the HPC and water AOC-values which might relate to the growth and survival of microbes, including enteric pathogens, are discussed.

2. Materials and methods

2.1. Water types included in the study.

The study includes the 5 leading brands of Norwegian bottled still waters: Brand 1, Brand 3 and Brand 4 (NMW in plastic bottles), Brand 2 (SW in plastic bottles), Brand 5a (SW/"artesian water" glass bottles) and Brand 5b (SW/"artesian water" plastic bottles).

2.2. Determination of the bacterial heterotrophic plate count at 22 ± 2 °C (HPC).

In order to account for putative inter-batch variations, analyses were performed on a minimum of 3 bottles from different production batches of each brand in the period October 2008 - November 2009. Each brand is produced at a single factory production site and from a single source water. Samples of 0.1 mL were removed in triplicate, diluted where required and spread onto R_2A agar (Oxoid, UK). Plates were packed in plastic to prevent drying and incubated in the dark at 22 ± 2 °C. The colony count was determined at 14 days.

Brand 1 water alone was available directly after bottling such that the development of the bacterial flora could be followed. Three bottles (1.5 L) were received at the laboratory within 24 h of bottling; samples were maintained at < 4 °C during transport. Upon arrival, each bottle was tested for the initial HPC. Thereafter two bottles were maintained at room temperature. A third bottle was refrigerated (4 ± 1 °C). Bottles were sampled at intervals using carbon-free pipette tips over a period of 6 months in order to follow the development of a HPC.

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2.3. Detection and enumeration of coliforms, E. coli, Enterococcus, Pseudomonas aeruginosa and spore-forming anaerobic sulphite-reducing bacteria.

Samples (250 mL), were filtered through a membrane filter (0.45 µM nitrocellulose) for cell capture. Filters were placed onto MacConkey agar (coliforms, *E. coli* and *Enterococcus*) or *Pseudomonas* cetrimide agar (*Pseudomonas aeruginosa*). Plates were incubated at 37 ± 1 °C and examined for colonies at 24 h and again at 48 h. Brilliance *E. coli*/coliform chromogenic medium (Oxoid) on which *E. coli* produces purple colonies and other coliforms pink colonies, was used to further characterize colonies appearing on MacConkey agar. Samples of 50 mL were filtered as above and incubated anaerobically at 37 ± 1 °C (48 h) on sheeps blood agar for the initial isolation of spore-forming anaerobic sulphite-reducing bacteria. Culture collection strains (*E. coli* ATCC 25922, *P. aeruginosa* DSM 9027, *Enterococcus faecium* ATCC 19434 and *C. perfringens* ATCC 13124) were used to confirm the suitability of the chosen methods. Three bottles representing different production batches of each bottle water brand were tested.

2.4. Determination of the optimal growth temperature and nutrient concentration of bacterial isolates.

Colonies from primary plates were streaked out on R_2A (relatively nutrient poor) and tryptone-soya agar (relatively nutrient rich). Plates were incubated at 4 ± 1 °C, 8 ± 1 °C, 22 ± 2°C and 37 ± 1 °C and read for growth (presence/absence) over a period of 14 days.

2.5. Detection and enumeration of fungi.

Parallels of 500-750 mL of bottled waters (2-3 bottles per brand) were filtered under low pressure through a 0.45 µM pore size nitrocellulose membrane (Millipore, MA). After filtering, the filter was placed onto Sabouraud Dextrose agar (Oxoid) modified to contain 100

μg/mL chloramphenicol (Sigma-Aldrich, St. Louis, MO). Plates were incubated at 22 ± 2 °C and examined regularly over a 7-day period. The remaining 500-750 mL of each bottle was filtered as above, with the exception that a 0.22 μM black, polycarbonate membrane was mounted on the nitrocellulose membrane (which here functioned as support). Fungal cells were stained using Fluoresence brightner, Calcofluor, (Sigma-Aldrich) as follows: after filtering, the mounted membrane was washed by filtering 200 mL of particle free water. Thereafter a 0.035 mg/mL solution of Calcofluor in 0.1 M Tris-HCL pH 9.0 was filtered to stain cells and the membranes were held in the dark for 30 min. Finally the unbound Calcofluor was removed by washing twice more (by filtration) with particle-free water. The entire membrane surface was examined for the presence of brilliant blue fungal cells and hyphae after mounting with the anti-fadent containing immersion oil Citifluor AF87 (Citifluor Ltd, London). An Olympus BX40 fluorescence microscope (Olympus, GmbH) fitted with an BP 330-385 nm exciter filter and BA 420 barrier filter combination was used. Images were transferred to a PC using an Olympus Cell Soft imaging system.

2.6. Colony-polymerase chain reaction (colony-PCR) of the 16S rRNA-gene.

All colonies adjudged to represent unike morphologies (in total 34) were subjected to PCR amplification of the 16S rRNA-gene. Amplification of an approximately 590 bp segment of the 16S rRNA-gene was performed as follows: the 50 μL reaction mixture contained: 10 μL 5× colorless Taq® Flexi Buffer (Promega, Madison), 4 μL 25 mM $MgCl_2$ (Promega), 1.3 μL deoxynucleotide mix (10 mM stock, Sigma-Aldrich), 0.5 μL of the primer pair 341F and 907R (Winter et al., 2005) to a final concentration of 1 μM (primers produced by Eurofins

MWG synthesis, GmbH), 2.5U GoTaq® Hot start Polymerase (Promega). A sterile needle or pipette tip was touched onto a well-isolated colony, and then dipped into the reaction mix to provide the DNA template for amplification. The PCR reaction was performed in a Palm-cycler (Corbett Life Science, Sydney) and was as follows**:** 34 cycles of: 95°C for 1 min., followed by 56 °C for 1 min. and 72 °C for 1 min. Cycling was completed by a final single elongation step of 72 °C for 30 min. in order to ensure that any remaining single-stranded DNA was fully extended prior to sequencing (Winter et al., 2005).

The presence of a single amplification product of the desired size was confirmed prior to sequencing by 1%-agarose gel electrophoresis of 4 μL of the finished reaction mix.

Sequencing was performed by a commercial laboratory (ABI lab., Blindern, Oslo) using the forward and reverse primers used in production of the PCR-product. Sequence similarity searches were conducted in April 2010 using the BLAST network service of the GenBank database to identify the nearest relatives of the partially sequenced 16S rRNA genes.

2.7. Colony-polymerase chain reaction of the fungal 5.8S gene and the noncoding Internal Transcribed Spacer regions ITS1 and ITS2

A forward primer binding to 3'-end of 18S rDNA and a reverse primer binding to the 5'-end of 25/28S rDNA of yeasts were used to amplify the intervening 5.8S gene and the noncoding Internal Transcribed Spacer regions ITS1 and ITS2 essentially as previously described (Trost et al., 2004). The total reaction volume of 100 μL contained the following final concentrations: 1X colorless Taq® Flexi Buffer (Promega, Madison), 1.5 mM $MgCl_2$ (Promega, Madison), 1 μM primer, 60 μM dNTPs (Sigma-Aldrich), 1 U GoTaq® Hot start Polymerase (Promega, Madison), colony material and water (ad 100 μL). After a denaturation

step of 3 min at 94°C, thirty-four cycles of PCR were performed (Palm-cycler, Corbett Life Science, Sydney) by using 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min., followed by a final extension at 72 °C for 10 min. Electrophoresis, sequencing and similarity searches were performed as described above for bacteria.

2.8. Identification and determination of the metabolic potential, of bacterial isolates using the Biolog GN2 microplate system (Hayward, CA).

Bacterial growth was swabbed from R_2A plate cultures (72 h; 22 ± 2°C) and suspended in the supplied suspension solution (GN/GP inoculating fluid) at OD_{530} = 0.13-0.14. The ability to completely homogenize colonies in the suspension solution was specified as an absolute requirement for performing the test. Colonies that did not disperse were not tested. The bacterial suspension was distributed (150 µL) into wells of the plate and incubated at 22 ± 2 °C for 96 h. Wells were scored for substrate metabolism visually (development of a violet colour due to reduction of tetrazolium to violet formazan) and spectrophotometrically using a Victor 1420 Multilabel Counter (Perkin Elmer, Turku, Finland). Following the lead of others (Péchy-Tarr et al., 2005) an optical density of at least 0.35 above the water control (well A1) value was set as the limit for a positive score. In addition, wells were only scored as positive when also a violet colour (as adjudged independently by the co-workers) was visible in the wells. A number of culture collection strains were included as control organisms. As specified by the manufacturer, isolates producing three or more postive reactions could be identified against the approximately 500 species in the GN2 database of the Biolog® MicroLog 4.2 plate identification system.

2.9. Measurement of Assimilable Organic Carbon (AOC).

The AOC content of bottled waters was measured on at least 3 occasions with different bottles from different production batches. AOC was measured essentially as previously described for tap water (Miettinen et al., 1999). In brief, 50 mL borosilicate tubes and their teflon corks were treated to remove organic carbon. Three parallel samples of 40 ml from each bottle were tested. In order to ensure that essential salts were not growth limiting, tubes were amended with 40 µL of a solution containing: 4.55 g $(NH_4)_2SO_4$, 0.2 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$ and 0.2 g NaCl in 1000 mL deionized water. This nutrient solution was also used in the growth yield control of the bacteria. In all experiments, the growth yield of the bacteria was controlled using a 50 µg acetate eq-C/L standard made in purified water (Milli-RX 45 water purification system; Millipore Corporation, USA). The AOC value of the control was corrected by subtraction of the value obtained in tubes containing only salts, water and the inoculum. All tubes (samples and controls) were pasteurized in a water bath at 65-70 °C for 30 min. Upon cooling, the test strains *Pseudomonas fluorescens* P17 (ATCC 49642) and *Aquaspirillum* NOX (ATCC 49643) were added from stock cultures to give individual start concentrations of 100-500 CFU/mL. The stock cultures were made by inoculating autoclaved, filtered (0.22 µM) river water amended with 1000 µg acetate eq-C/L (Merck, Germany). Stock cultures were incubated at temperature of 22 ± 2 °C until the maximum counts on R_2A agars were reached, and thereafter stored at 4 ˚C for no more than one year. After inoculation, tubes were incubated in the dark at 22 ± 2 ˚C and, after suitable dilution, samples were spread in duplicate at intervals (5, 7 and 11 days) onto R_2A to determine the maximum plate count reading which is converted to the AOC-value. The concentrations of AOC are presented as the sum of AOC-P17 and AOC-NOX based on the strains growth yields on acetate, and using the formula:

AOC µg/L = [(*P. fluorescens* CFU/mL)(1/yield*) + (*Aquaspirillum* sp. NOX CFU/mL)(1/yield**)] (1000 mL/L)

*4.1 × 10^3 P-17 CFU/mL equals 1 µg of P-17 AOC/L and **1.2 × 10^4 NOX CFU/mL equals 1 µg of NOX AOC/L

2.10. Survival of Escherichia coli in bottled waters.

E. coli ATCC 25922 was grown on R_2A agar for 18-24 h at 37 ± 1 °C. Thereafter, cells were washed 2 times in purified water by resuspension and centrifugation in order to remove residual organic carbon. Pelleted cells were finally resuspended in purified water at a concentration corresponding to a MacFarland 0.5 standard (~ 1 × 10^8 CFU/mL), diluted 10^{-1} in water and added to previously unopened bottles to achieve a concentration of 100 CFU/mL. Immediately before inoculation with *E. coli*, samples from bottles were spread on R_2A agar to determine the HPC (as described above), and on MacConkeys agar (Oxoid) at 37 ± 1°C to ensure that coliforms were not present. Bottles were stored at 22 ± 2 °C, and assayed daily for the presence of *E. coli* by plating on MacConkeys agar. Bottles were followed until 0.1 mL samples on 3 consecutive days produced no colonies. The time up to the first day of this 3 day series was taken as the survival time of *E. coli* in each bottle. Three bottles of each brand were included in the test. Correlation analyses (Pearson product-moment) between mean parameter (AOC, HPC, survival-time) values for each brand were performed.

2.11. Statistical analysis.

Statistical analyses were performed on log-10 transformed data. Results are given together with standard deviation from the mean. Correlation analysis (Pearson product-moment) was used to determine the degree of association between variables. One-way

analysis of variance (ANOVA) with least significant difference (lsd) t-tests (post-hoc) was used to exam variability in log-10 transformed experimental data. The probability level at which statistical analyses were accepted as significant was < 0.05.

A dendrogram was constructed for the bacterial isolates based on the results of the Biolog GN2 analysis. Data were coded in a binary notation by using 1 (substrate metabolized) and 0 (substrate not metabolized). All characters were assigned equal weights. A percentage similarity matrix was obtained by computation. The matrix was subjected to a clustering procedure where junctions at decreasing similarity levels were established based on the unweighted pair-group average (UPGMA) method. Data analyses were performed using a commercially available multivariate statistical package (Kovach Computing Sevices, Wales, UK).

3.0 Results

3.1 The presence of enteric indicator bacteria and pathogens

No colonies of any kind were produced from any brand on Cetrimide or blood agars under the conditions specified. On MacConkey agar only Brands 2 and 4 produced colonies (< 5/250 mL). These were Gram-negative rods which gave colourless colonies both on the original MacConkey plates and after subculturing onto *E.* coli/coliform chromogenic medium, and were thus not coliforms. All of the brands tested met the current microbiological requirements for bottled water quality (Helse- og omsorgsdepartementet, 2004) with respect to their content of enteric indicator organisms and specified pathogens.

3.2 The heterotrophic bacterial plate count (HPC) at 22 ± 2 °C.

Bottled water brands produced large HPC consisting of between 3-6 unique colony morphologies, where usually 1-2 morphologies dominated. Colonies from Brand 3 were typically large and coloured (yellow, orange) and grew quickly. A number of these were identified as *Sphingomonas* species (see *3.2* below). In contrast, colonies from Brand 1 were minute and generally white and none became visible before approximately one week of incubation. Major types included facultative methylotrophs such as *Methylobacterium* and *Methylibium* (Table 1). Mean HPC values as presented in table 1. Geometric mean colony counts ranged from 1200 - 28000 CFU/mL. Brand 5 waters have a shelf-life of two years. Only plastic bottles (Brand 5b) 1-6 months after bottling could be obtained, whereas only glass bottles (Brand 5a) which had stood 7- 18 months were available. Owing to the differing

ages of the glass and plastic brand 5 bottles at the time of testing, attempts were not made to compare their HPC in detail. It was noted that the populations were generally different, but that some colony types appeared in both waters.

Three 1.5 L bottles of Brand 1 were followed from production by repeated sampling with carbon-free tips over a period of 6 months. Samples (1 mL) taken immediately after bottling did not produce a single colony from any bottles. New samples taken 72 h after bottling again produced no colonies. For parallel bottles maintained at 22 ± 2 °C, the highest HPC was reached at around 7 days after bottling before declining to less than 1000 CFU/mL at 90 days. The highest values reached in the two bottles which were maintained at 22 ± 2 °C were 30000 CFU/mL and 28000 CFU/mL, respectively which are similar to the other peak values obtained with other Brand 1 bottles included in the study (geomteric mean = 28000, Table 1). A bacterial population also developed in the refrigerated bottle, and this is in keeping with tests (see *3.3*) of constituent strains' temperature range for growth. However, the maximum colony count (2200 CFU/mL, 90 days) never reached the levels obtained for the bottles maintained at room temperature and fell to 200/mL in the 6 months sample. The bacterial flora which developed in both bottles was dominated (90-100%) by small (< 1mm) white colonies (Brand 1.5 – *Variovorax* sp., Table 2), and a slightly larger (< 2mm) rough colony morphology (Brand 1.6 – *Rhodoferax* sp., Table 2).

3.2 Identities of the bacteria grown from bottled waters based on sequencing of the 16S rDNA gene and Biolog GN2 microplates.

With a single exception, all of the 34 unique colony morphologies grown from bottled waters could be assigned to known phylogenetic groups based on sequencing of the 16S rRNA-gene (Table 2). Neither colonies nor purified DNA preparations of isolate 2.6 produced

a PCR-product, and the identity of this strain was not investigated further. Of the 33 sequences to which a taxonomic group was assigned, 3 represented Gram-positive taxa. The remaining strains divided between betaproteobacteria (55%), and alphaproteobacteria (36%). All of the betaproteobacteria could be assigned to the order *Burkholderiales.* Although the brands appear pristine with respect to enteric indicator bacteria and pathogens specified in the legislature (section *3.1*), genera previously associated with a variety of opportunistic infections of immunocompromised hosts, notably *Sphingomonas* (Angelakis et al., 2009) and *Methylobacterium* (Rice et al., 2000) were among the HPC colonies (Table 2). Isolates were also characterised and, where possible, identified based on their metabolic capabilities. A dendrogram was constructed using the presence/absence of metabolism of 95 substrates included in the GN2 plate (Fig. 1). Thirty of 34 isolates were amenable to testing (see criteria in *2.8*). Of these, 50% produced no color change in the GN2 plate indicating non-metabolism of the included substrates under the test conditions. The control *Pseudomonas* and *E. coli* strains, and particularly a number of putative *Sphingomonas* isolates clustered by virture of metabolism of a large number of the included substrates. Only 8 isolates (Table 2) as well as the control strains gave the required 3 or more postive reactions for entry into the MicroLog database for identification. Table 2 presents also a comparison of identifications based on sequencing studies and the Biolog GN2 system. Most of the strain designations based on sequencing of the 16S rRNA gene are not covered by the GN2 database, and thus the nearest named species is included in the table. In only one instance (B1.1, *Acitenobacter iwoffii*) is the identification considered close enough to be acceptable to the microlog database. Here sequencing studies suggest the non-database species *Methylibium petroleiphilium*. In most cases where the microlog database included the sequencing-defined strain, the strain appeared amongst the two most likely identifications at the genus level.

3.3. Trophic status (growth on R_2A versus TSA) and optimal growth temperatures.

Bacteria were grown on the relatively nutrient-poor medium (R_2A) and the nutrient rich TSA. It was found that 74% of the isolates grew best on R_2A, and 26% grew as well, or better (one strain) on TSA. Approximately 80% of the isolates grew best at 22 ± 2 ˚C, whereas 20% grew as well or better at 37 ± 1 °C. As many as 91% of the strains were adjudged to show some growth at 4 ± 1 °C - 8 ± 1 °C ˚C, whereas only 24% grew at 37 ± 1 °C ˚C. No isolates had 4 ± 1 °C - 8 ± 1 °C ˚C as optimum temperature. Isolates which showed growth at these low temperatures would, in keeping with generally accepted criteria, be considered psychrotolerant (Morita, 1975).

3.4 Fungal isolates.

Fungi were detected only sporadically and in low numbers. No fungi were detected in any bottles by cultivation or Calcofluor staining of Brands 1 and 2. Brand 4 produced no colonies on agar. However, one sample (500 mL) showed a small number (< 10) of budding yeasts on the Calcofluor stained membrane (results not shown). One sample of the 5b brand produced 2 colonies of a yeast identified as *Cryptococcus magnus* (closest relative/closet named relative FN400938.1, 100% similarity, max score 926). Inoculation of this isolate onto R_2A, non-nutrient agar and water agar gave colonies on all media at 4 ± 1 ˚C and no growth at 37 ± 1 ˚C. Best growth was obtained at 22 ± 2 ˚C on R_2A. These results are in keeping with a psychrotolerant and oligotolerant metabolism. Some *Cryptococcus* sp. (particularly *C. neoformans*) are considered important pathogens for humans whilst other (eg, *C. gattii*) are considered as emerging pathogens (Galanis et al., 2010). Given the medical importance of the genus and the existence of standardised susceptibility breakpoints for some *Cryptococcus*

species, attempts were made to determine the antibiotic resistance profiles of the *C. magnus* isolate. However, the strain grew first after 5 days in the standard test RPMI 1640/glucose medium (results not shown) placing it beyond the scope of accepted susceptibility testing methodology. Brand 3 showed a small number of yeasts both by cultivation, and Calcofluor staining. Analysis of the entire contents (1500 mL) of one bottle produced 11 colonies of yeasts. Of these, 10 presented with a black central region. These were identified by sequencing as *Aureobasidium pullulans* (AJ244231.1, closet relative/closet named relative, 100% similarity, max score 913). The other yeast was putatively identified as *Heliotiales* sp bjelland (closet relative/closet named relative AY011014.1, 99% similarity, max score 841)..

3.5 AOC content of bottled waters and correlations with the HPC.

Mean AOC values are presented in table 1. The geometric mean of AOC values ranged from 13.5 - 115 µg/L (Brands 1, 2 respectively). AOC values were subjected to ANOVA to determine variability between brands. The differences between the brands were not significant (F (5, 26) = 2.18, p = 0.095). Post-hoc analysis showed that only brand 1 and 2 means were significantly different (p = 0.043). Brand-related differences in the growth responses of the two AOC test organisms *P. fluorescens* P17 and *Aquaspirillum* NOX were also noted. The AOC values of Brands 2 and 4 were based on good, concomitant growth of both assay strains P17 and NOX. By contrast only NOX grew in the Brand 1 and Brand 3 waters. The relationship between AOC and HPC values for individual bottles (n = 27) of all brands was moderately positive and significant (Table 3).

3.6. Survival of Escherichia coli in bottled waters and correlation with AOC.

E. coli became non-viable in < 10 days in all bottles of low-AOC brands tested (Brands 1 and 3) but persisted for longer periods (> 26 days) for all bottles of the high AOC brands tested (Brands 2 and 4). For all brands taken together, the relationship between mean survival times and mean AOC-values was strongly positive and significant. The correlation between *E. coli* survival times and HPC was also positive and significant (Table 3).

4.0 Discussion

4.1 The presence of enteric indicator bacteria and pathogens

This study sought to determine the quality of the leading brands of Norwegian bottled waters with respect to microbiological quality criteria given in the current legislation (Helse- og omsorgsdepartementet, 2004). Neither enteric indicator species nor *Pseudomonas aeruginosa* or spore-forming anaerobic sulphite-reducing species were detected in any of the brands. As such the situation seems promising by comparison with recent similar studies of bottled waters in some other nations in with comparable legislation (Venieri et al., 2004; Baumgartner and Grand, 2006, Mavridou et al., 2008).

4.2 The heterotrophic bacterial plate count (HPC) at 22 ± 2 °C.

The growth of HPC species for Brand 1 followed from the bottling stage appears to be typical for bottled waters in general (Loy et al., 2005 and references therein). Ie, there is a rapid increase in the HPC from a low density indigenous population reminiscent of the source water, to high concentrations a few days after bottling. All bottles of all brands tested gave large HPC. HPC values for bottles tested ranged from 1200 CFU/mL (Brand 3) to 28000 CFU/mL (Brand 1) and this indicates that some brands can support larger bacterial populations than others. Given the large bacterial numbers in bottled waters, the salient point for the consumer is the health significance, if any, of the particular microbes present.

Although no upper limit is set on the HPC of bottled waters which develops after bottling, we know little about its composition, and the numbers and types of carbon sources in the water supporting its development. It is now generally accepted that the HPC alone does not directly relate to health risks for the population in general. However, the implication for infections of immunocompromised patients in the general community remains unclear and there is insufficient data for its evaluation (Expert Meeting Group Report, 2003; Pavlov et al., 2004). Specific strains of microbial species that may be a part of HPC microbiota can cause infection in certain vulnerable people (e.g., the immunocompromised and those with indwelling or intravenous catheters). Furthermore, the HPC and its composition can indicate changes in water quality and it should be evaluated as a trigger for further investigation (Expert Meeting Group, 2003).

4.3 Identities of the bacteria grown from bottled waters based on sequencing of the 16S rDNA gene and Biolog® GN2 microplates.

There are numerous studies documenting the size and composition of bottled water HPCs using different media for initial isolation and differening identification techniques (see below). Comparisons of various available identification systems have shown that the same isolate will be identified differently according to the database used and many identifications have been made employing clinical systems for which the database was not appropriate for environmental strains (Expert Meeting Group, 2003 and references therein). A case in point are the differing identifications obtained using sequencing studies and the biochemistry-based Biolog® GN2 system in the present study (Table 2). The microlog database (aproximately 500 species) covers only a fraction of the new genera and species defined by genotypic studies. Furthermore, the Biolog® system, and other systems such as API (BioMerieux, France)

specify the use of nutrient rich media for inoculum production, short incubation times and high incubation temperatures, all of which would preclude the majority of bottled water isolates listed in Table 2. It should thus be kept in mind that the results presented in Table 2 are based on a modification of the standard method. In a comparable study of 103 bacterial isolates from a deep aquifier (McCarthy and Murray, 1996), 48 isolates could not be identified and the remaining isolates could only be tentatively identified using the microlog database. The sequencing approach (Table 2) suggests that the R_2A-HPC is dominated by betaproteobacteria (55%), and alphaproteobacteria (36%). All of the betaproteobacteria could be assigned to the order *Burkholderiales.* The composition of the HPC is reminsicent of a recent study of bottled waters in which non-cultivation techniques were used (Loy et al., 2005). In that study, betaproteobacteria represented > 80% of the taxonomically aligned strains, and the genera *Polaromonas, Aquabacterium* and *Hydrogenophaga* made up 20-86% of the FISH-defined species.These genera are also among those identified in the current study (Table 2). Results from both studies are in seeming contrast to many previous ones where analysis of the HPC using usually more nutrient-rich media (eg, PCA) and biochemical identification systems have found larger populations of gammaproteobacteria (Loy et al., 2005 and references therein). For example, randomly chosen colonies from PCA cultures of seven newly purchased bottled brands were identified. Four of seven samples contained *Pseudomonas fluorescens* (as sole gammaproteobacterium) where it alone accounted for 26-100% of the total bacterial count (Armas and Sutherland, 1999). In several studies, use of the API 20NE system and other phenotypic approaches did not allow the identification of a large proportion of bottled water isolates (Morais and da Costa, 1990; Guillot and Leclerc, 1993). The relative high proportion of gammaproteobacterial species probably arises from a bias in the testing based on cell culture (Loy et al., 2005) and the non-suitability of biochemical tests systems and their databases for environmental strains (Expert Meeting Group, 2003) . The use

of R_2A and genotyping in the present study whilst still only identifying a fraction of the microbiota shown to be present by non-culture techniques, supports the proposed dominance of alpha- and betaproteobacteria in pristine bottled waters (Loy et al., 2005).

The finding of a preponderance of psychrotolerant strains which also prefer R_2A to TSA accords with the origin of the brands from protected groundwaters arising from glacial melt. None of the isolated bacterial strains were closely related to any primary pathogen. However, some of the genera found such as *Methylobacterium* and *Sphingomonas* (Table 2) have been reported as opportunistic pathogens of immunocompromised patients. *Sphingomonas* spp. particularly *S. paucimobilis* have been associated with community-acquired and nosocomial infections including bacteremia, meningitis, catheter-related sepsis and diarrheal disease (Ammendolia et al., 2004; Kilic et al., 2007). Similarly Methylobacteria, particularly *Methylobacterium* sp. have been associated with bacteremia of a child with lymphoma, catheter and systemic infections (Rice et al., 2000; Sanders et al., 2000). These observations should be seen in light of that groups targeted for marketing have included infants and the immune-compromised (Papapetropoulou, 1998).

4.4 AOC content of bottled waters and correlations with the HPC

There is still much debate as to the reasons for the development of a HPC in waters after bottling (see Leclerc, 2003). One parameter that has not been investigated for bottled waters is AOC. The value of AOC lies in the observations that the HPC in distribution systems remains low when the AOC value approaches 10 µg/L at the treatment plant (Van der Kooij et al., 1999), and that levels between 50 - 100 µg/L seem to limit coliform proliferation (see LeChevallier et al., 1999). However, the AOC test has obvious weaknesses. It is not a direct measure of total available carbon and it does not indicate the amount or types of carbon

available to a population of microbes, particularly if these are trophically different from the AOC assay strains. Evidence that this may be the case for many bottled water isolates comes from the Biolog® analysis. Of the isolates amenable to testing, 50% produced no color change in the GN2 plate indicating non-metabolism of the included substrates under the specified test conditions. By contrast, the *Pseudomonas* strains (including P17 used in the measurement of AOC) and *E. coli* utilized a large number of the included substrates and cluster together (Fig. 1). Several of the bottled water isolates (Table 2) belong to genera (*Methylibium, Methylobacterium*) which are facultative methylotrophs, and can employ other substrates for growth than those measured in the AOC assay. In two cases (B 1.7; B 2.1 - Table 2) bacteria belonging to these genera dominanted the HPC from the particular bottles from which they were isolated. Notwithstanding the HPC - AOC relationship was significant and moderately positive, indicating that devlopment of a bottle HPC may in part be explained by bacterial utilization of substrates contributing to measured AOC values. *E. coli*-survival times in bottled waters showed a significant and strongly positive correlation with both AOC and the HPC (Table 3). *E. coli* quickly became non-viable in low AOC brands (1, 2 and 5a,b) but persisted practically unchanged in numbers in high AOC brands (2 and 4) for several weeks before declining, indicating that these waters are hospitable for the maintenance but not growth of the test strain. *E. coli* did not grow in any of the brands, but one interpretation of its persistence in Brands 2 and 4 is that there is sufficient AOC to maintain a basal cellular metabolism. AOC values between 50-100 µg/L have been equated with increased numbers of coliforms (LeChevallier et al., 1999). Furthermore, it has been found that growth of *E. coli* in water correlated with AOC values. Specifically it was found that *E. coli* O157 grew at AOC values above about 180 µg/L, but not at 10 µg/L, (Vital et al., 2008). The significant, positive relationship between *E. coli* survival time and the bottle HPC might be due to fixation of biomass by these bacteria or the creation of a microenvironment in which the survival of *E.*

coli is promoted. The findings are important in that *E. coli* is the major indicator of fecal contamination in the bottled water directive (Helse- og omsorgsdepartementet, 2004). Protracted survival times of this species increase the chances for its detection. The *E. coli* –survival time might also reflect the survival times of enteric bacterial pathogens such as *Salmonella* sp, and the nutritionally diverse *Pseudomonas aeruginosa*, and this will in the future be investigated.

4.5 Fungal isolates from bottled waters

The numbers, types and resistance profiles of fungi causing infections have increased dramatically in recent years. Several species of fungi are capable of infecting healthy hosts causing diseases ranging from mucosal to life-threatening systemic infections. In addition, there are an increasing number of severe fungal diseases by commensal or fully saprophytic species in immunocompromised hosts (Enoch et al., 2006). Notwithstanding, we know little about the role of water in the dissemination of fungal pathogens. There is for example, considerable debate as to whether *Aspergillus* and *Fusarium* spp infections are transmitted by water (Expert Meeting group 2003 and references therein). Molds were not found in the Norwegian brands tested. Yeasts were detected only in low numbers. Thus, the brands tested do not seem to be good vectors for fungal dissemination. *C. magnus,* isolated from Brand 5b was both psychrotolerant and grew on water-agar. The genus *Cryptococcus* contains the important pathogen *C. neoformans* which has been associated with a severe menigitis. Other members of the genus including *C. albidus* and *C. luteolus* are also reported to cause meningitis, lung abscess and fungemia. In addition the species *C. gattii* is a well-characterized emerging pathogen (Galanis et al., 2010). Thus, the detection of *Cryptococcadaceae* in food and water require attention. However, a search of the literature seems to implicate *C. magnus*

only in a single reported case of infection, namely otitis externa in a cat (Kano et al., 2004). *A. pullulans* was isolated from Brand 3 and has also previously been connected with low temperature pristine environments (D'Elia et al., 2009). The black pigmented centre (chlamydospores) of the colonies fits descriptions in the literature (Hawkes et al., 2005) and supports the genotype-based identification. The presence of *A. pullulans* deserves further comment. Although this species is widely distributed in the environment, it has been reported to cause opportunistically a variety of infections, including periotonitis, cutaneous infections, pneumonia and meningitis. There have also been episodes of systemic infections (Hawkes et al., 2005). To our knowledge, neither *C. magnus* nor *A. pullulans*, have previously been reported in bottled waters. That infections by both species have been reported underscores the need to extend analyses of bottled waters to include fungi.

The five leading brands of Norwegian bottled waters seem to meet good standards of hygiene. None of the named pathogens and indicator organisms specified for testing in current European quality standards were found. Identification of indigenous yeasts and the HPC revealed the presence of species which have been associated with opportunistic infections, but which are not regarded as primary pathogens. However, some brands were more hospitable to the survival of the enteric *E. coli* and the development of high HPC than others, and this may be related their content of AOC. These brands might represent more hospitable environments to microbial contaminants than others.

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