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ORIGINAL PAPER

S.M. Soini · K.T. Koskinen · M.J. Vilenius
J.A. Puhakka

Effects of high and fluctuating pressure on microbial abundance and activity in a water hydraulic system

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Abstract The effects of high and fluctuating pressure up to 220 bar on microbial growth and activity were determined in a pilot-scale water hydraulic system. An increase in the pipeline pressure from 70 to 220 bar decreased the total and the viable cell number in the pressure medium from $2.2(\pm0.5)\times10^5$ to $4.9(\pm1.5)\times10^4$ cells/ml and from $5.7(\pm2.8)\times10^4$ to $1.3(\pm0.7)\times10^4$ cfu/ml, respectively. Microbial attachment in the non-pressurised tank of the hydraulic system increased with increasing pipeline pressure [from 1.0(±0.3) to $3.8(\pm2.7)\times10^5$ cells/cm^2 on stainless steel]. The phosphatase, aminopeptidase and β-glucosidase activities in the pressurised medium were between 0.02 and 1.4 µmol/lh (V_{max}) and decreased in response to increasing pipeline pressure. The α-glucosidase activity was detected only at 70 bar and the glucuronidase activity only occasionally. Based on principal component and cluster analyses, both the pressure applied and the original filling water quality affected ubstrate utilisation patterns. This study demonstrated the capability of freshwater bacteria to tolerate high and fluctuating pressure in a technical water system. Microbial survival was due to attachment and growth on the surfaces of the non-pressurised components and the nutrient flux released by cell lysis in the pressurised components. In summary, high pressures in water hydraulic systems do not prevent potential microbiologically related operational problems.

Introduction

Water hydraulics is a relatively new technology that uses water instead of oil as a pressure medium. Oil-based hydraulic systems have negative environmental impacts through leakages, whereas water is innocuous. Water is readily available and the cost is low as compared to oil. Water hydraulics has several different applications, including process and food industries, fire protection and underwater systems. Microbial accumulation in the system may reduce the efficiency, introduce thermal resistance and accelerate corrosion. Biofouling decreases filter and valve lifetimes by clogging the components.

Microbiologically, the water hydraulic system is an extreme environment characterised by high and fluctuating pressure, high fluid flow velocity (up to 6 m/s) and low nutrient concentrations. Microbial growth and accumulation in pilot- and full-scale systems has been reported (Soini et al. 2000, 2002a). The effects of fluid flow velocity and water quality on the microbiology of these systems has been described (Soini et al. 2002b).

Pressure tolerance is a widely documented feature of bacteria in saline, especially deep-sea, environments (e.g. Yayanos 1995; Kato and Bartlett 1997; Kato et al. 1998; Takami et al. 1999). Many deep-sea bacteria isolated are either piezophilic or piezotolerant and are unable to grow at temperatures above 20°C (Kato and Bartlett 1997). The typical temperature range in water hydraulics is 25–50°C. The phenomenon of pressure tolerance has been described in environments such as the food processing industry (Simpson and Gilmour 1997; Benito et al. 1999; O'Reilly et al. 2000). To our knowledge, the phenomenon has not been demonstrated in technical freshwater systems.

This study focuses on a hydraulic system where tap water is used as a pressure medium. The pressure in the system can fluctuate from 0 to 1,000 bar (over atmospheric) and the fluctuation cycles experienced by the microbes are numerous and frequent during system operation. The aim of this study was to determine microbial survival, growth, activity and attachment in a pilot-scale water hydraulic system that uses elevated pressures (up to 220 bar) and has a pressurised cycle duration of 2 s every 5 min.

S.M. Soini (✉) · J.A. Puhakka
Institute of Environmental Engineering and Biotechnology,
P.O. Box 541, 33101 Tampere, Finland
e-mail: sari.soini@tut.fi
Tel.: +358-3-31153519, Fax: +358-3-31152869

K.T. Koskinen · M.J. Vilenius
Institute of Hydraulics and Automation (IHA),
Tampere University of Technology, Finland

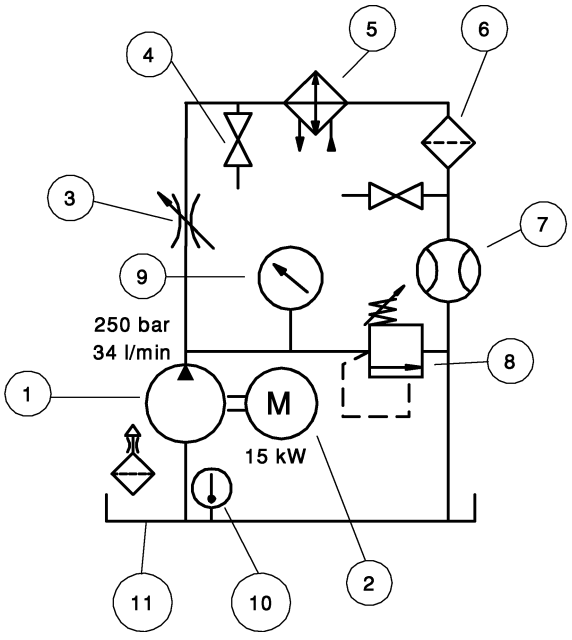

Fig. 1 The pilot-scale water hydraulic system. *1* Pump, *2* motor, *3* restrictor valve, *4* gate valve/sampling, *5* heat exchanger, *6* filter, *7* flow meter, *8* pressure regulator, *9* pressure meter, *10* thermometer, *11* vented reservoir

Materials and methods

The pilot system

A pilot-scale water hydraulic system was used in the experiments (Fig. 1). The system consisted of a plunger-type pump (CAT 3507), a motor (15 kW), stainless steel piping, a tank [medium density polyethylene (PE-MD); 200 l], a cooler, a filter [pore size 20 µm, polypropylene (PP), Pall Profile II, Pall, UK] and a breather filter (pore size 10 µm, Hydac, Finland). The pressure in the pipeline was controlled by a pressure control valve. Stainless steel (AISI 316) and PE-MD slides (26×75 mm) were placed into the tank to monitor microbial attachment. A slide holder on the bottom of the tank kept the slides in an upright position.

Experimental set-up and sampling

The collector slides were rinsed with technical ethanol and the system was disinfected by flushing with sodium hypochlorite (1 mg/l) for 2 h prior to the experiments. No cells were detected on the collector slides and there were no viable cells in the pressure medium at the beginning of the experiments (day=0). Tap water was used as a pressure medium with an initial volume of 180 l. The temperature of the pressure medium was set to 35°C. The volumetric flow was 32 l/min, corresponding to a flow velocity of 1.9 m/s in the pipeline. Pressures of 70, 160 and 220 bar were used in the experiments. The length of pressure cycle was 2.2 s and the interval between the cycles, 5 min. The pressure medium was at atmospheric pressure between the cycles. Water samples were taken aseptically from the tank and the pipeline after the pressure cycle (no. 4: gate valve/sampling in Fig. 1). Collector slides (in duplicates) were removed from the tank aseptically and analysed as described below.

Chemical analyses and enumeration of bacteria

The dissolved organic carbon (DOC) concentration was determined according the Finnish standard SFS-EN 1484 (Finnish Standards Association 1997). The samples were filtered (Whatman Puradisc, 0.45 µm) and analysed with a Shimadzu TOC-5000 carbon analyser. The heterotrophic viable plate counts were determined on R2A medium (Reasoner and Geldreich 1985) by a spread plate method. The plates were incubated at 35°C for 3 and 7 days. The total numbers of bacteria were determined by staining the filtered samples (0.2 µm, polycarbonate, Millipore) with DAPI (4′,6′-diamidino-2-phenyl-indole) and counting the cells with an epifluorescence microscope (Zeiss Axioskop 2). At least 20 randomly selected fields were counted from each sample. The collector slides were stained with DAPI prior to counting the bacteria.

Enzyme activity assays

The enzymatic activities in the pressure medium (α- and β-glucosidase, phosphatase, glucuronidase and aminopeptidase) were determined as the hydrolysis of their respective MUF (4-methylumbelliferyl)- and MCA (4-methylcoumarinyl-7-amide)-based substrates (Sigma). The glucosidase, phosphatase and aminopeptidase substrates were measured as indicators of the overall microbial activity and to provide complementary results to culture physiology studies (Biolog assays, described below). The glucuronidase activity was an indicator of coliform bacteria (Shadix and Rice 1991). Stock solutions of the substrates were prepared to a concentration of 5 mM in dimethylsulfoxide (DMSO). Working solutions (2.5 mM–1 µM) were diluted with deionised autoclaved water. For the assay, 20 µl of substrate solution was added to 200 µl of sample in a 96-well sterile titre plate. The final concentrations were between 0.1 and 500 µM. The plates were incubated at 35°C in the dark for 7 days. The initial and released fluorescence intensities were measured with a microplate reader (Wallac Victor, 1420 Multilabel counter). The excitation and emission were 355 nm and 460 nm, respectively.

Biolog assays

Biolog ECO microplates (Biolog, Hayward, Calif.) were used to study the substrate utilisation patterns of the microbial communities in the water hydraulic system. Each well was inoculated with 150 µl of sample water. The plates were incubated at 35°C in the dark for 7 days. The optical densities (λ=590 nm) were determined with the microplate reader.

Statistical analyses were carried out using SYSTAT9 (SPSS, Science Software, Gorinchem, The Netherlands). The bacterial numbers were log10 transformed and examined using ANOVA and *t*-tests. Statistical significance was assumed at a P value of <0.05. The enzyme kinetics (V_{max}) was calculated using SigmaPlot version 7 and the Enzyme Kinetics Module 1.1 (SPSS). The Biolog data was analysed as follows: the colour response of the control well (R) was subtracted from each of the response wells (C) [$C–R$]. The average well colour development (AWCD) values of the plates were calculated as follows: $AWCD=\{[\Sigma(C–R)]/n\}$, where n is the number of substrates. The data was normalised by dividing the raw difference values ($C–R$) by the AWCD of the plate (Garland and Mills 1991). The relationships among the samples were determined by principal component analysis (PCA) and cluster analysis (SYSTAT9; SPSS).

Results

Microbial growth

The development of microbial numbers during the initial operation of the water hydraulic system was as described previously (Soini et al. 2000, 2002b). These included the decrease of the total cell numbers (DAPI counts) in the pressure medium during the 1st day of the experiments

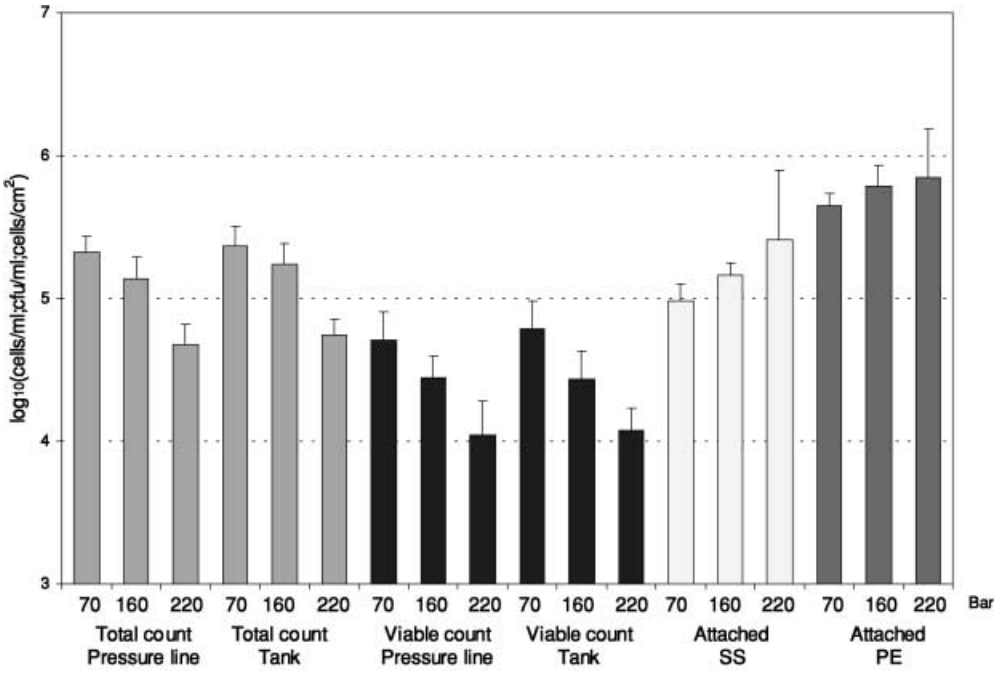

Fig. 2 The total cell numbers (DAPI) and the plate counts (R2A 7 days at 35°C) in the pressure medium, and the attached cell densities (DAPI) in the tank during the constant phase (7–21 days of operation, *n*=7). *SS* Stainless steel, *PE* polyethylene

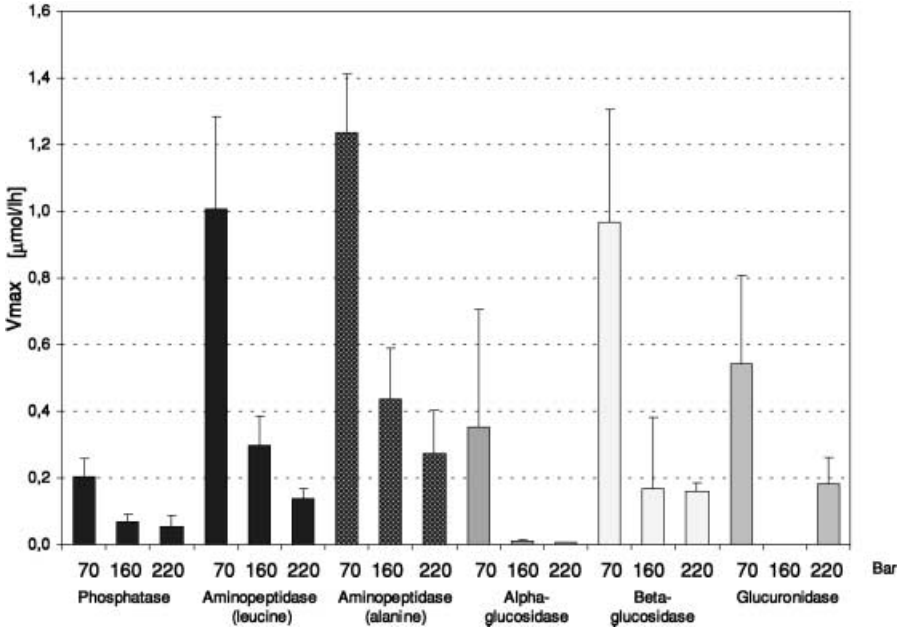

Fig. 3 The enzymatic activities (V_{max}) in the pressure line of the pilot-scale water hydraulic system during the constant phase (7–21 days of operation, *n*=7). The aminopeptidase substrate is given in parenthesis

and a gradual increase towards a constant cell number that was reached on days 9–16. The numbers of viable heterotrophic bacteria in the pressure medium increased 4–5 log in 3 days and thereafter stabilised to a constant level (results not shown).

The average DAPI counts in the pressure line during the constant phase were $2.2(\pm0.5)\times10^5$, $1.5(\pm0.5)\times10^5$ and $4.9(\pm1.5)\times10^4$ cells/ml for the pressures 70, 160 and 220 bar, respectively. The plate counts in the pressure line during the constant period were $5.7(\pm2.8)\times10^4$, $3.0(\pm1.1)\times10^4$ and $1.3(\pm0.7)\times10^4$ cfu/ml for the pressures 70, 160 and 220 bar, respectively (Fig. 2). The results show that the increase in the pipeline pressure significantly decreased the total and the viable cell numbers in the pressure medium (P=0.01 and 0.0, respectively). The cell numbers in the non-pressurised tank and in the pressure line within each individual experiment were similar. The DOC consumptions during the operation were 0.2(±0.04), 2.7(±0.09) and 1.4(±0.43) mg/l for the pressures 70, 160 and 220 bar, respectively. The higher consumptions at 160 and 200 bar resulted from higher original DOC levels in the pressure medium.

Microbial attachment in the tank started within 2 days. The cell densities on the collector slide surfaces

Table 1 The number and type of substrates utilised in selected samples from the water hydraulic system. Biolog ECO microplates, *PRE* Pressure line, *tank* non-pressurised tank

Sample / Substrates utilised	Amino-acids (6)[b]	Carboxylic acids (9)	Phosphorylated compounds (2)	Carbohydrates (6)	Polymers (4)	Esters (1)	Amines (3)
70 bar[a] PRE 21 days	2	5	–	4	4	1	2
70 bar tank 21 days	3	3	2	1	1	–	1
160 bar PRE 7 days	3	3	–	1	1	–	–
160 bar PRE 14 days	3	1	1	–	1	–	–
160 bar PRE 21 days	2	–	1	–	–	–	–
160 bar tank 7 days	3	1	2	–	–	–	1
160 bar tank 14 days	3	3	1	–	1	–	1
160 bar tank 21 days	3	–	1	–	1	–	–
220 bar PRE 7 days	3	3	1	–	2	–	1
220 bar PRE 14 days	3	1	–	–	2	–	–
220 bar PRE 21 days	3	1	–	1	1	–	–
220 bar tank 7 days	3	3	1	1	–	–	1
220 bar tank 14 days	3	2	1	1	1	–	–
220 bar tank 21 days	2	3	–	1	–	–	–

[a] Pressure level, sample and the duration of the experiment
[b] Total number of substrates tested within the group

gradually increased until a constant cell density was reached on days 4–7 (results not shown). The cell densities during the constant phase were $1.0(\pm0.3)\times10^5$ to $8.7(\pm4.8)\times10^5$ cells/cm^2 (Fig. 2). The increase in the pressure increased microbial attachment in the tank of the water hydraulic system. The increase was significant on stainless steel (P=0.003). Polyethylene was more readily colonised than stainless steel (P=0.0).

Microbial activity

The phosphatase and the aminopeptidase activities in the pressure medium followed the numbers of viable bacteria and, during the constant phase, varied between 0.02 and 0.28 µmol/lh and 0.1 and 1.4 µmol/lh (V_{max}), respectively. The aminopeptidase activity was higher than that of the phosphatase (Fig. 3). The α-glucosidase activity in the pressure medium was detected only with the lowest pressure. The β-glucosidase activity was similar to the leucine-aminopeptidase. Glucuronidase activity was frequently detected with 70 bar and occasionally with 220 bar. The increase in the pipeline pressure decreased the enzymatic activities (P=0.0 for aminopeptidases and β-glucosidase, P=0.27 for α-glucosidase). The activities in the pressure line and in the tank of the hydraulic system were similar within each individual experiment.

Substrate utilisation patterns

The utilisation of 31 different substrates (in triplicate on Biolog plates) was tested in each sample. The microbial communities of the water hydraulic system utilised 10–58% of the substrates tested. The substrates utilised on the Biolog microplates were mainly amino acids, other organic acids and phosphorylated compounds (Table 1). The number of substrates utilised generally decreased during the experiment. The PCA of the substrate utilisation patterns distinguished the different pressures as well as the different sampling points (Fig. 4). The microbial community from the 70 bar pressure line consumed a wider variety of substrates than the communities from the other parts of the system. The first principal component (PC1) accounted for 68%, and the second (PC2), 16% of the total variance in the data. The cluster analysis (hierarchal clustering, SYSTAT) revealed a similar pattern as the PCA, differentiating both the pressure applied and the sampling point. The principal component and the cluster analyses showed that both the applied pressure and the original filling water quality affected substrate utilisation patterns.

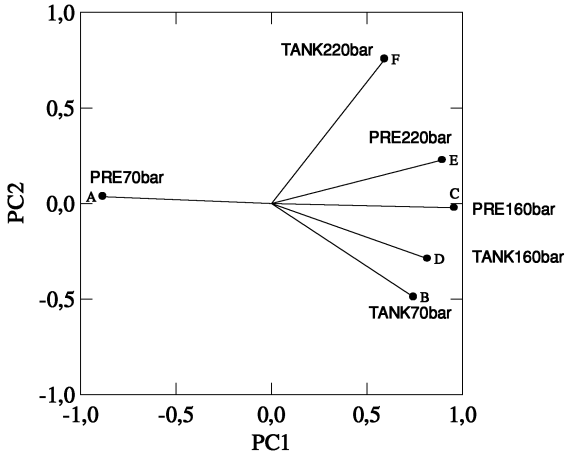

Fig. 4 The effect of pressure on the substrate utilisation patterns as determined based on the principal component analysis. *A–F* Sample codes, *PRE* pressure line, *tank* non-pressurised tank

Discussion

Microbial growth and attachment have been documented in low- and medium-pressure water hydraulic systems (Soini et al. 2000). The pressure in a high-pressure application can fluctuate from 0 to 1,000 bar (over atmospheric) and the duration of the pressurised cycle can range from a few seconds to several minutes. The systems are operated without changing of the pressure medium for several months, thus the pressure fluctuation cycles experienced by the microbes in the medium are numerous and frequent. In this work, microbial growth, attachment and the effects on catabolic activities under high and fluctuating pressure up to 200 bar in a water hydraulic system were demonstrated.

An increase in the pipeline pressure significantly decreased the total and the viable cell numbers in the pressure line and in the non-pressurised tank of the water hydraulic system. The growth pattern and the cell numbers were similar to our previous studies under low pressure (Soini et al. 2002b). The microbial attachment in the non-pressurised tank increased along with the increasing pipeline pressure. Microbial survival in the system under pressure fluctuation was due to attachment and growth on the surfaces of the non-pressurised components. Bacterial lysis may have occurred during the pressure cycle, releasing nutrients into the pressure medium. This could have supported the growth of viable bacteria reducing the overall effect of pressure fluctuation. Also, increase in microbial attachment may have resulted from the increased nutrient flux. However, no significant decrease in cell numbers or increase in DOC was observed after the pressure cycle. Polyethylene was more readily colonised than stainless steel. Similar results have been obtained by Schwartz et al. (1998) in studies of drinking water distribution system biofilms. Contrary to this, Pedersen (1990) found no difference in the cell densities on steel and polymer surfaces. Also, in water hydraulic systems, the most favourable material for microbial attachment may vary depending on the application.

Phosphatase and aminopeptidase activities increased significantly in 3 days and thereafter stabilised, as did the numbers of viable heterotrophic bacteria in the pressure medium. The higher aminopeptidase activity indicated pressure-induced release of amino acids into the medium. Enzymatic activities have been related to bacterial numbers and the nutritional status of the environment (Hoppe et al. 1988; Chróst 1991). In this study, the enzymatic activities/cell, as estimated by dividing the measured enzymatic activities with sample cell numbers, decreased with increasing pressure. Thus, the decrease in activity was pressure-induced and not only due to the decrease in the cell number with increasing pressure. Glucoronidase activity was detected at 70 and 220 bar, indicating the presence of coliform bacteria in water (Shadix and Rice 1991). The results indicate that coliforms may survive in the pressure medium under the pressure fluctuation.

The PCA of the Biolog patterns with ECO plates distinguished both the different samples (pressure line and non-pressurised tank) and the three pressure levels. The PCA showed that the pressure and the original filling water quality affect substrate utilisation. Also, the results indicated pressure-induced changes in the microbial community structure. Amino acids were the most readily utilised group of substrates, in accordance with the enzymatic activity results. The limitations of using the Biolog method in studying differences in microbial community structure and function include the required cell density in the sample and the high substrate concentrations in the wells (Konopka et al. 1998). Therefore, only a limited range of the microbes in the community, which may not be the dominant ones, influence the substrate utilisation patterns (Haack et al. 1995; Verschuere et al. 1997; Smalla et al. 1998). Biolog ECO plates were introduced to better characterise heterotrophic communities and they have been shown to perform as well as the GN plates that were designed to characterise the physiology of bacterial isolates (Choi and Dobbs 1999). Due to these method limitations, the substrate utilisation patterns are only indicative of community differences in the water hydraulic system.

In conclusion, this study showed the capability of freshwater bacteria to tolerate high and fluctuating pressure with moderate reductions in numbers and activity. Thus, high pressures in water hydraulic systems do not prevent microbiologically related operational problems. The pressure tolerance of freshwater bacteria is probably not limited to hydraulic systems and has potential applications in other fields of biotechnology.

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