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(54) **Title:** AUTOMATED PROCESS FOR DETECTING PATHOGENIC ORGANISMS IN WATER

(57) **Abstract:** The present invention relates to an entirely automated process for detecting the presence of pathogenic organisms in a water sample. The invention also relates to an entirely automated device for detecting the presence of pathogenic organisms in a water sample, for carrying out this process.

**« Automated process for detecting pathogenic organisms in water »**

The present application claims the priority of the U.S. Provisional Application N°60/307,171.

The present invention relates to the general technical domain of the detection of pathogenic organisms in water.

In particular, the present invention relates to an entirely automated process for detecting the presence of pathogenic organisms in a water sample. The invention also relates to a device for detecting the presence of pathogenic organisms in a water sample, for carrying out this process.

The presence of pathogenic agents in water supply networks, such as drinking water or industrial water networks, engenders real risks to the health of human beings. Water supply networks thus, mostly, contain pathogenic organisms, such as viruses, bacteria, protozoa, fungi, amoebae or worms, which are capable of causing various diseases in humans. Among these diseases, mention may be made, inter alia, of cholera, typhoid fever, diarrhoea, dysentery, legionellosis or gastroenteritis.

Among the pathogenic organisms responsible for such diseases, mention may be made more particularly of the Cryptosporidium (parvum) and Giardia Lamblia protozoa both of which originate from human and animal fecal waste and which are a major cause of gastroenteric diseases, the Toxoplasma gondii and Naegleria fowleri protozoa which also originate from human and animal fecal waste and which cause various serious diseases, Cyclospora which cause diarrhoea, and coliform bacteria, in particular Escherichia coli, which are found throughout the biosphere and the detection of which is important since they are considered not to be typical pathogenic agents, but to be organisms which indicate water contamination, as they cause diseases only at very high concentrations. In the context of the present

invention, for the purpose of simplification, all organisms which indicate water contamination and all pathogenic organisms actually responsible for diseases in humans will be included in term "pathogenic organisms". Among pathogenic organisms responsible for diseases, mention may also be made of the bacteria Salmonella, including the serotypes S. enteridis, S. enterica, S. typhi and S. paratyphi, which are the cause of 70% of the bacterial diseases of food origin, in particular typhoid fever and paratyphoid fever, and which are the cause of a large number of deaths, Heliobacter pylori, which is an organism linked to the cause of at least 75% of all stomach ulcers and to two types of stomach cancer, and which is also the cause of a large number of deaths, and Legionella, which is found in natural areas and also in water heating systems, which causes Legionnaires' disease, and also viruses of human and animal origin, and in particular hepatitis A viruses, viruses of the Norwalk type, rotaviruses, adenoviruses, enteroviruses and rheoviruses. Norwalk viruses in particular cause sporadic and epidemic gastrointestinal diseases with diarrhoea. Approximately 200 000 cases thereof per year are seen in the United States.

Given the presence of various pathogenic agents in all water supply networks, the latter should be controlled and possibly disinfected before the water which comes from these networks can be considered to be drinking water. With regard to waste water or cooling water supply networks, and also energy production networks, in particular nuclear energy production networks, it is sometimes necessary to analyse the water and even to disinfect it before it can be reused or discarded into the surrounding environment.

The detection of the presence of pathogenic organisms, in particular the detection of slowly growing highly pathogenic organisms, which are the most difficult to detect, thus represents a major challenge in the spheres of water treatment, of water distribution and of the

quality control of water. The conventional techniques for detecting pathogenic agents, developed to date, have various drawbacks: they are not used continuously or in real time, they have a relatively low sensitivity with respect to pathogenic organisms and they prove to be long and expensive in terms of material and personnel.

A first known technique uses the counting of microorganisms which develop after culturing the sample on various selective nutrient media. This technique is simple but has considerable risks of error and of artefact (poor specificity of morphological criteria, absence of development of slowly-growing microorganisms, exponential growth on the nutrient medium of bacterial strains which are in reality in the lag phase of growth in the sample). Its response time is, in addition, generally longer than 24 hours.

A second technique, using the measurement of one or more enzymatic activity/activities enables the rapid quantification of populations of living microorganisms (microorganisms which can be cultured and/or microorganisms which are in viable form but which cannot be cultured) (WO 90/100983). This technique in particular allows a set of populations to be monitored, but does not enable a very fine degree of specificity to be obtained.

A third technique, using immunological probes, has a response time which is frequently longer than 24 hours, and often lacks sensitivity and specificity (artefacts due to cross reactions may be observed).

Other recent techniques use specific oligonucleotide probes which are generally labelled so as to allow their detection after hybridization to their targets. Two main types of oligonucleotide probe have been developed: probes with DNAs (or the mRNAs which correspond to them) as the target, and probes with rRNAs (ribosomal RNAs) as the target. However, DNA or mRNA probes, although potentially very specific, have the drawback of being

relatively insensitive due to the low number of DNA (or mRNA) copies per microbial cell.

A need thus exists to develop a process and a device for detecting the presence of pathogenic organisms in a water sample, said process and device not having the drawbacks of the techniques of the prior art and thus making it possible to carry out the on-line detection of several pathogenic microorganisms in water in a very short period of time (less than 6 hours), less expensively and with high sensitivity and specificity, making it possible to get a detection limit of 1 organism in 100 ml of water sample analysed and thus to respect the standards imposed on drinking water quality.

The present invention satisfies this need. The Applicant has thus discovered, surprisingly, that this could be carried out with the aid of a process and a device, controlled entirely automatically, which can be used on an industrial scale, preferably taking as a basis the technique of amplifying DNA or RNA using a polymerase chain reaction (PCR), or a similar technique.

The process according to the present invention is thus entirely automated and contains no manual step. It is based on the detection of DNA or RNA sequences specific for certain pathogenic microorganisms. The automation of certain single steps has already been described in the prior art, but the automation of all of the sequential steps of the process according to the present invention has never been envisaged until now. Thus, the extraction on a solid support and the purification of nucleic acids present in a liquid sample have already been automated and have been described in patent applications WO 00/75623 and WO 97/10331. Similarly, the automation of nucleic acid amplification carried out by polymerase chain reaction (PCR), and also the detection of organisms by fluorescence, have already been envisaged in patent application WO 00/33962. However, the problems encountered in carrying out the automation of the five sequential steps of the

process according to the present invention have thus never been overcome until now.

A subject of the present invention is thus a process for detecting the presence of pathogenic organisms in a water sample, comprising the succession of the following steps:
concentration of the pathogenic organisms,
breaking open of the cells of the pathogenic organisms,
concentration of DNA and/or of RNA from the cells,
at least one nucleic acid amplification, and
a qualitative and/or quantitative detection of DNA and/or RNA representative of the water contamination by said pathogenic organisms;
characterized in that all of the sequential steps of the process are controlled fully automatically using a control logic system.

The water analysed in the process for detecting the presence of pathogenic organisms, according to the present invention, is advantageously taken from supply networks, such as drinking water networks, industrial or waste water networks, cooling water supply networks, or energy production networks, in particular nuclear energy production networks.

Advantageously, according to the present invention, the pathogenic organisms detected are preferably bacteria, viruses, fungi and/or protozoa.

Advantageously, according to the present invention, the starting volume of the water sample is of more than 50 milliters, preferably from 100 ml to 10 litres, more preferably from 2 to 3 litres.

According to the present invention, nucleic acid amplification is necessary in the detection process in order to be able to detect a minimum of one pathogenic agent, and preferably from 1 to 5 pathogenic agents, in the water sample analysed.

Advantageously, according to the present invention, the nucleic acid amplification is carried out by a real-time polymerase chain reaction (PCR). PCR is a commonly used amplification technique. The PCR technique (Rolfs *et al.*, 1991), requires the choice of pairs of oligonucleotide primers bordering the fragment which must be amplified. The general principles and the conditions for carrying out nucleic acid amplification by PCR are well known to those skilled in the art and in particular are described in US patents 4,683,202; 4,683,195 and 4,965,188.

Other similar techniques for amplifying nucleic acids (DNA and/or RNA) can advantageously be used as an alternative to PCR (PCR-like) using pairs of primers of nucleotide sequences. The term "PCR-like" is intended to denote all methods using direct or indirect reproductions of nucleic acid sequences, or in which the labelling systems have been amplified, these techniques of course being known to those skilled in the art. Mention may, in particular, be made of the SDA (Strand Displacement Amplification) technique (Walker *et al.*, 1992), the TAS (Transcription-based Amplification System) technique described by Kwoh *et al.* (1989), the 3SR (Self-Sustained Sequence Replication) technique described by Guatelli *et al.* (1990), the NASBA (Nucleic Acid Sequence Based Amplification) technique described by Kievitis *et al.* (1991), the TMA (Transcription Mediated Amplification) technique, the LCR (Ligase Chain Reaction) technique described by Landegren *et al.* (1988), the RCR (Repair Chain Reaction) technique described by Segev (1992), the CPR (Cycling Probe Reaction) technique described by Duck *et al.* (1990) and the Q-beta-replicase amplification technique described by Miele *et al.* (1983). Some of these techniques have since been improved. Thus, according to the present invention, specific detection of pathogenic agents can be carried out using such techniques for amplifying nucleic acids at high speed.

In comparing the technique of amplification by PCR or by a similar technique, such as NASBA, with conventional techniques for detecting pathogenic agents, such as cell culture, the Applicant has discovered that it is possible to reduce the length of time required for the assay from a few weeks or a few days to a few hours. In addition, PCR amplification or a similar technique is easy to carry out and the initial costs, like the subsequent costs for carrying out the PCR, prove to be considerably lower than the costs generated for carrying out cell culture techniques. In addition, a PCR can be used to identify a specific form of the pathogenic agents which are found in the water. It can thus be used to detect the infectious state of an organism, proving the presence or absence of DNA or of RNA specific for the pathogenic agent. The PCR amplification according to the invention also makes it possible to obtain a process with high sensitivity, advantageously greater than 1 picogramme, and even more advantageously greater than a few femtogrammes of nucleic acids.

The Applicant has also discovered that the use of PCR amplification (or a similar technique) followed by in situ specific real-time detection, for instance by fluorescence, produces analytical data relating to the detection and to the identification of the organisms more rapidly and more accurately than any other detection system based on biochips. Biochips make it possible to detect a much greater number of pathogenic species than that detected using on-line PCR, but, on the basis of known pathogenic agents (which can be more effectively tested as groups), the real-time PCR is much more effective and gives a better specificity than standard hybridized biochips. In addition, chip hybridization, which allows the microorganisms to be brought into contact with a DNA and/or RNA probe, takes several hours, and even often includes overnight incubation, which is significantly longer than the real-time amplification of the DNA or of the RNA.

A large variety of primers can be used to carry out the nucleic acid amplification, preferably by PCR, according to the present invention, some of these primers already being available in databases or in the literature, certain others having already been developed for specific organisms.

In a particular embodiment of the present invention, a nested PCR is used, by dividing the PCR into two steps, in order to obtain maximum selectivity with respect to the pathogenic agents whose detection in the water is being sought. The first PCR is carried out with the entire sample (typically several tubes of 100 - 200 microlitres with only a few DNA molecules) using a set of degenerate primers and employing a standard thermocycler machine. After amplification for a few cycles (for example from 10 to 15 cycles) at high temperature (75 to 100°C), the fluid which then contains at least several thousands of copies of each DNA can be divided into a certain number of vials in a second PCR machine, using a means of automatic sampling and distribution such as a robot. A second set of primers is then added to each vial and a second nucleic acid amplification is then carried out using on-line detection, for example by fluorescence. It will thus be possible to use the first amplification to amplify nucleic acids of groups of pathogenic agents using a set of universal (degenerate) primers, while the second amplification will make it possible to determine accurately the species present in the sample analysed, using a set of primers which are specific to the pathogenic agents whose detection is being sought. In this particular embodiment of the present invention, detection is carried out while carrying out the second PCR whereas no detection is carried out while carrying out the first PCR which only amplifies nucleic acids.

While in the medical domain the analysis of blood samples of a few microlitres to a few millilitres is involved, in research and control relating to the

environment, samples having volumes which can range up to several tens of litres are usually analysed. However, the direct techniques for breaking open the cells of the organisms cannot be carried out on such large volumes, and the pathogenic organisms, whose detection is being sought according to the process of the present invention, must be concentrated down to volumes of less than 100 ml before being able to be given the remainder of the treatment. In addition, environmental standards impose that an organism must be proved to be absent in 100 ml of water sample analysed. The difficulty in concentrating pathogenic organisms derived from samples of the order of a litre of water is losing as few organisms as possible while at the same time reducing the volume of the sample down to a volume of about one hundred ml.

Advantageously, according to the present invention, the concentrating of the pathogenic organisms is carried out by precipitation, coprecipitation or precipitation by inclusion. Such a concentrating of organisms starting from sample volumes of about one litre thus makes it possible to have a highly sensitive process and to respect the standards imposed on water quality. Specifically, the greater the volume of a sample, the greater the sensitivity and the more accurate the analytical data. In addition, the detection process according to the present invention makes it possible to detect the presence of less than 10 organisms in initial sample volumes of 100 ml to 10 litres, preferably 2 to 3 litres. Furthermore, although the instruments of the competition use either filtration or capture by magnetic beads as a first concentration step, precipitation by inclusion is more effective in terms of trapping a wide variety of organisms and more economical, and allows highly contaminated samples to be treated. According to the present invention, the concentrating of the pathogenic organisms includes the automatic sampling of water samples analysed.

Even more advantageously, according to the present invention, the concentrating of the pathogenic organisms is carried out in the presence of divalent metal and carbonate ions advantageously chosen from the group consisting of calcium carbonate, strontium carbonate and barium carbonate. The reaction medium is brought to a relatively high temperature, preferably of about 40 to 60°C, so as to allow the rapid formation, in a few minutes, of a precipitate containing various pathogenic agents, simply by settling out and without additional gravitational force. In order to obtain efficient settling out, the reagents such as the divalent carbonate ions are not added to the reaction medium all at once, but are added step by step. After formation of the precipitate, onto which the various pathogenic agents of the water sample adsorb or are trapped within , the supernatant liquid is taken off and the precipitate is dissolved in an acid, such as formic acid. The reaction medium containing the dissolved precipitate is then neutralized with a buffer agent.

Advantageously, according to the present invention the step for breaking open the cells, which follows that for concentrating the pathogenic organisms, is carried out by grinding on agitated beads. For the purposes of the present invention, the expression "breaking open the cells" is intended to mean rupture or lysis of the cells from the pathogenic organisms, intended to release the content, and in particular the nucleic acids, of these cells. The operating conditions for this step are adjusted so as to avoid any modification of the nucleic acids. The step for breaking open the cells according to the present invention is carried out using analysed sample volumes of about 10 to 100 ml, preferably of 50 ml, and has been designed to preferably take place in a semi-continuous system using beads agitated by a strong source of energy so as to rupture the cells from the organisms by means of direct collisions and impacts. The particles for the splitting open, according to the present invention, are retained in

the splitting chamber (cracking chamber), whereas the cells ruptured subsequent to the collisions generated by the particles, and the nucleic acids thus released from the cells, are set free from the cracking chamber, thus enabling the grinding of the beads to function in a semi-continuous manner.

Advantageously, according to the present invention, the beads are agitated by ultrasound (generally leading to rather small DNA fragments of around 100 - 200 bp), by mechanical shaking or by mechanical vibration, preferably for approximately up to ten or so minutes. Even more advantageously, according to the present invention, the beads are agitated by mechanical shaking or vibration, using for example a rotating setup, generally leading to a size of DNA fragments of between 200 - 2000 kB. The increase in temperature created during the agitation and collision of the particles also appears to contribute to the effectiveness of the breaking open of the cells. The addition of chemical agents or of enzymes, such as proteases, may also promote the splitting open of the cells, in particular by digesting the cell walls.

Advantageously, according to the present invention, during the breaking open of the cells, or subsequent to the breaking open of the cells and prior to the concentrating of DNA and/or of RNA, at least one chaotropic agent, advantageously a guanidine salt, such as guanidine thiocyanate, and at least one adsorption-modifying agent, advantageously an alcohol such as ethanol, are added to the reaction medium containing the cells, preferably with the aid of pumps and valves, in order to facilitate the extraction of DNA and/or of RNA from the cells and in order to inactivate the nucleases, which are enzymes capable of destroying the nucleic acids. The guanidine salt, such as guanidine thiocyanate, is preferably added, according to the present invention, at a concentration of more than one mole per litre. Even more advantageously, according to the present invention, a mixture of chaotropic agent and

adsorption-modifying agent is added to the reaction medium containing the cells from the pathogenic organisms.

Advantageously, according to the present invention, the step for concentrating DNA and/or RNA, which follows that for breaking open the cells, is carried out by reversible adsorption of the nucleic acids onto a solid support containing an adsorbent material, followed by elution of these acids.

Care is necessary to avoid trapping of nucleic acids at the walls of the system by strong or irreversible adsorption. Especially metal surfaces such as stainless steel tubes, rings or containers have a high tendency to trap the nucleic acids and are thus advantageously avoided. Thus, all adsorptive metal parts are advantageously avoided or eluted after DNA contact.

According to the present invention, the step for concentrating DNA and/or RNA corresponds to the extraction and purification of DNA and/or RNA.

According to the present invention, the adsorbent material is advantageously a vitreous material, preferably comprising surfaced hydroxy groups, or a silica-based compound.

According to the present invention, the elution of the nucleic acids is advantageously carried out by washing the absorbent material using at least one solvent containing monovalent ions, advantageously chosen from the group consisting of sodium thiocyanate, potassium thiocyanate and EDTA. The solvent according to the invention is thus advantageously a complexing agent made of monovalent ions.

The Applicant has thus discovered, surprisingly, that the elution of the nucleic acids from the adsorption support cannot be carried out without the prior conversion of the complex of nucleic acids and of divalent ions, formed during the first step of concentrating the organisms, into a complex consisting of nucleic acids and of monovalent ions. Specifically, the complex of nucleic

acids and of divalent ions is strongly attached to the solid adsorption support and cannot be desorbed and then eluted by simply washing using a solvent. The desorption, followed by the elution, of the nucleic acids is only made possible by adding a complexing agent made of monovalent ions. Such an agent thus makes it possible to exchange the divalent ions, attached to the nucleic acids, against monovalent ions and thus to form a new complex which may be desorbed much more easily than the nucleic acid/divalent ions complex. After carrying out the ion exchange, a solvent is then added to finalize the elution of the nucleic acids. This solvent must be compatible with the amplification step which follows the step for concentrating the nucleic acids, and is advantageously chosen from the group consisting of water or a diluted buffer.

Advantageously, according to the present invention, the process may also contain a step for concentrating the volume in order to attain volumes of about one hundred µl up to less than 1 ml, advantageously less than 100 µl, before carrying out the nucleic acid amplification and subsequent to the step for concentrating the nucleic acids.

Advantageously, according to the present invention, the step for detecting the pathogenic organisms, which follows that of nucleic acid amplification, is an on-line (real-time), in situ, detection. Even more advantageously, according to the present invention, the step for detecting the pathogenic organisms is carried out by fluorescence, by luminescence or by mass spectrometry. It may also be carried out by other detection techniques well known to those skilled in the art, such as those of hybridization on chips or of enzymatic amplification.

Even more advantageously, according to the invention, the detection is carried out in situ by fluorescence. In order to obtain a detection signal, CyberGreen or another double-stranded intercalation fluorophore may thus be added during the amplification step, making it possible to indicate the success of the

amplification by a strong increase in fluorescence during the cycle. Specifically, the primers labelled using the fluorophores hybridize specifically with the target DNA of the sample. Since the PCR amplification reaction generates a large number of copies of the target DNA, it is thus possible to obtain a quantification of the intensity of the fluorescence signal, which is directly correlated with the initial amount of target DNA in the sample analysed.

In order to obtain multispecific detection, i.e. detection specific for several pathogenic agents, in a single vial and in order to increase the specificity with respect to these pathogenic agents if this is required, molecular beacons or similar tracers can be added to the various vials used during the amplification. Using standard instrumentation, it is possible to detect up to four different species per vial.

Advantageously, the detection process according to the present invention may also contain a prior filtration step, before carrying out the step for concentrating the pathogenic organisms. Such a filtration may prove to be useful when analysing samples of crude water or of surface water, particularly samples contaminated and loaded with troublesome substances such as sand.

Advantageously, the detection process according to the present invention may also contain a prior step for treating the water sample by heat shock and/or by a nutrition stress, with for example glycerol or ethanol as carbon source, and/or by adding a gene-inducing chemical compound in order to induce the RNA of specific genes and to detect the viable pathogenic organisms in said water sample. Specifically, a heat shock (an increase in the temperature for a few moments) or the addition of a chemical compound induces a set of proteins thus allowing an RT (reverse transcriptase) reaction to be carried out as a first step, when the first PCR is carried out, which thus makes it possible to obtain a signal specific for viable organisms. The RT reaction allows the transformation of RNA

into DNA. If it proves to be necessary, DNAases can be added in order to destroy the native DNA. Specificity is obtained by inducing a DNA which is specific to a high degree.

5 Advantageously, according to the present invention, a heat shock is performed by heat pulse for less than 3 hours, advantageously at a temperature of 30 to 50°C.

Advantageously, according to the present invention, a gene-inducing chemical compound is added. Such a compound 10 makes it possible to induce the RNA of specific genes. The compound is advantageously a nutrient element, such as lactose, or a targeted inducer, such as IPTG (Isopropyl-(beta)-D-thiogalactopyranoside). This chemical compound allows a large increase in the level of specific RNAs in 15 the cells. The duration of the induction depends on the organism, but is typically approximately between 2 minutes and 1 hour. The heat-induced proteins, such as dnaJ, grpE, hscB, hslJ, hslV, htpG, htpX, htrB, htrC, pphA, pphB, rpoE, degP, groEL, clpA, dnaK, inpA/B and other heat-shock 20 proteins (for example of the small HSPs, HSP 60, HSP 70, HSP 90, HSP 100 and ubiquitin classes), are effective targets.

According to the present invention, inducing the RNA of specific genes can also be carried out by UV-25 irradiation, by using toxic substances as e.g. cis-platinum, anisomycin, tunicamycin, arsenites, by osmotic shock, by ethanol or by a minimal medium with for example glycerol as carbon source. For the purposes of the present invention, the expression "minimal medium" is intended to 30 mean a medium which contains the minimal amount of chemicals needed for the organisms to survive.

The induction is direct proof of the viability of the cell. Furthermore, RNA can thus be detected in the form of many copies with greater sensitivity compared to the 35 DNA. Although the detection of DNA is important, since the DNA is present in each cell in a fixed amount and it is, therefore, easy to make a correlation between the DNA and

the number of cells, the RNA can, itself, be induced at various levels, thus making it possible to detect the viability of the pathogenic organisms.

Advantageously, according to the present invention, the gene-inducing chemical compound is lactose or IPTG so as to induce the lac Z RNA. The lac Z operon, which has a unique sequence in a certain number of pathogenic organisms, in fact constitutes a useful target. The duration of induction is approximately a few minutes.

Advantageously, according to the present invention, the detection of the viable pathogenic organisms in a water sample is carried out after having obtained a DNA signal representative of the presence of pathogenic organisms in said water sample. Thus, in a particular embodiment of the present invention, all of the sequential steps of the process for detecting the presence of pathogenic organisms are first carried out in order to conclude on the possible contamination of the water sample analysed. Then, if a DNA signal is obtained, all of the sequential steps of the process are again carried out but a prior step for treating the water sample by heat shock and/or by adding a gene-inducing chemical compound is added in order to induce the RNA of specific genes. The DNA signal is then compared to the RNA signal and reliable viability data can then be obtained.

Advantageously, according to the present invention, the control logic system consists of at least one computer, for instance of the PC type, which controls all of the steps. The control logic system thus makes it possible to control the various steps of the process and, at the end of the process, to receive the analytical data relating to the detection of the organisms.

Advantageously, according to the present invention, the analytical data relating to the detection of the organisms are automatically transferred to a central control system. Advantageously, this central control system

consists of at least one computer. This central control system will be able to group together, analyse and control all of the analytical data, for example using databases, and incorporate these data into mathematical models which
5 will make it possible to predict the level of contamination of the water networks analysed, the type of microorganism present in these networks and also the survival rate of these microorganisms.

Advantageously, according to the present invention,
10 the process can function continuously and autonomously for a period of time greater than or equal to one day, preferably from three days to seven days. For example, this period of time depends on the number of reagents used.In addition, a given water sample can be assayed and analysed
15 several time a day using the detection process according to the present invention. To do this, specific DNA sequences (primers) are used and, if necessary, new primers can be developed. The assay duration for a test cycle using the process according to the present invention is optimized at
20 a period of time which is on the order of the doubling time of most organisms (2 to 6 hours).

A subject of the present invention is also a device for detecting the presence of pathogenic organisms in a water sample, comprising:
25 a unit for concentrating the pathogenic organisms (1),
a unit for breaking open cells from the pathogenic organisms (2),
a unit for concentrating DNA and/or RNA (3),
a unit for amplifying nucleic acids (4), and
30 a unit for qualitatively and/or quantitatively detecting DNA and/or RNA (5) representative of the water contamination by said pathogenic organisms;
characterized in that it also comprises:
at least one a control logic system (6) which controls
35 fully automatically the sequential treatment steps carried out by units (1) to (5),
pumping means (7),

automatic sampling and distribution means (8), and
a central control system (9).

Advantageously, according to the present invention, the unit for amplifying the nucleic acids (4) consists of at least one thermocycler polymerase chain reaction machine. According to the present invention, the PCR machine carries out sequential amplification cycles, each cycle being carried out at a given range of temperature and for a given period of time. If necessary, according to the present invention, the amplification unit consists of two thermocycler polymerase chain reaction machines in order to obtain maximum selectivity with respect to the pathogenic agents whose detection in the water is being sought. The two machines are then used sequentially. The transfer of the nucleic acids from one machine to the other is carried out using automatic sampling and distribution means (8) and a control logic system (6) which controls these means.

In a particular embodiment of the present invention, a thermocycler machine with integrated on-line detection is used to detect the amplification of the specific DNA. This type of machine thus consists of a thermocycler PCR machine with an integrated on-line detection chamber. In a particular embodiment of the present invention, when carrying out a nested PCR, the second PCR is carried out in this type of machine, the first PCR being carried out in a standard thermocycler polymerase chain reaction machine. Such a machine can be acquired from certain companies such as Biorad, Cepheid, Perkin Elmer or Roche Molecular Biochemicals. In this particular embodiment of the present invention, amplification unit (4) and detection unit (5) are grouped together in the same machine.

Advantageously, according to the present invention, the unit for concentrating the pathogenic organisms (1) is a reactor made of metal, comprising a system for introducing the water sample, advantageously consisting of valves, a system for introducing various reagents,

advantageously consisting of tubes, of valves and of rotary or cam-operated pumps, an agitation system, a system for evacuating the supernatant liquid, advantageously consisting of tubes and of valves, a system for evacuating
5 the precipitate, advantageously consisting of valves and of pumps and advantageously placed at the bottom of the reactor, and a heating system advantageously consisting of a heating jacket (sheath) placed around the reactor.

The reactor according to the present invention is
10 made of metal, advantageously of stainless steel, in order to obtain a high resistance to the chemical reagents introduced into said reactor. The reactor advantageously has a volume of 100 ml to 10 litres, preferably from 1 to 3 litres. The tubes are, themselves, advantageously made of
15 steel or of plastic.

The introduction of the water sample according to the present invention is carried out automatically via valves which are opened and closed with the aid of the control logic system (6). According to the present
20 invention, the introduction of the various reagents required for concentrating the pathogenic organisms is carried out using tubes, valves and rotary or cam-operated pumps, said valves and pumps also being activated by the control logic system (6). The agitation and the heating are
25 also controlled by the control logic system (6). The internal temperature of the reactor is advantageously controlled using sensors.

Advantageously, according to the present invention, the unit for breaking open the cells (2) contains a bead
30 grinder. Advantageously, the beads used are chemically inert and should be sufficiently solid to cause the cells to rupture. The beads used according to the present invention are advantageously small beads, such as polymer beads, for instance plastic, the diameter of which can
35 range from a few mm to a few tens of μm. In order to improve the splitting open of the cells from the pathogenic organisms, the beads are agitated by a strong source of

energy. The possible sources of energy for agitating the beads can be ultrasound sources, shakers of the rotary shaker type, devices of the Ultradurax type, devices of the sonotrode type or forceful vibrators.

5 Advantageously, according to the present invention, the unit for concentrating DNA and/or RNA (3) contains one or more solid support(s) containing a reversible adsorption material for nucleic acids, advantageously a vitreous paste. The nucleic acids can thus be concentrated, for 10 example using a porous absorption column containing a vitreous material, preferably comprising surfaced hydroxy groups, or a silica-based material, which will allow the reversible adsorption of the nucleic acids to the solid support.

15 Even more advantageously, according to the present invention, the solid supports consist of microtitration plates containing multiwells, advantageously 60-, 96- or 384-well plates which, themselves, contain adsorbent materials. Each well corresponds to a reversible adsorption 20 microcolumn.

Even more advantageously, according to the present invention, the wells of the microtitration plates are used only once and are moved automatically in order to receive the liquid sample originating from the unit for breaking 25 open the cells from the organisms. Compressed air may be injected in order to carry the fluid into the wells. The plate is referenced with respect to a system of XY coordinates, X corresponding to the axis along the width of the plate and Y corresponding to the axis along the length 30 of the plate. The automatic movement of the plate, and consequently that of the wells which are attached to the wells, takes place along the X and Y axes, for example using the arm of an automaton. The automatic movement can be activated by the control logic system according to the 35 present invention. In a particular embodiment of the present invention, the automatic movement is carried out using an automaton, which is controlled using a control

logic system (6) advantageously consisting of a computer. Advantageously, the automaton has an arm which can move around according to an XYZ frame of reference.

Advantageously, according to the present invention, the wells of the microtitration plates (or tube racks) can be protected against evaporation with a drop of fluid having a low specific density with a transparent lid.

After elution of the various nucleic acid samples present in the wells used during the adsorption, all the liquid samples are grouped together in order to be able to carry out the subsequent steps for amplifying the nucleic acids and for detecting the pathogenic organisms.

Advantageously, according to the present invention, the detection unit (5) consists of a fluorescence detector. Advantageously, the fluorescence detector comprises an optical unit such as an electronic camera which is able to record the photons coming from the fluorophores and to convert them into electrons. Subsequently, the electronic picture is read out and transferred to the control logic system (6).

The functioning of the device according to the invention will be more clearly understood upon reading the description given hereinafter with reference to the attached Figure 1, which diagrammatically illustrates a particular embodiment of the present invention.

The detection device according to the present invention comprises at least one unit for concentrating the pathogenic organisms (1), a unit for breaking open the cells from the pathogenic organisms (2), a unit for concentrating (adsorbing then eluting) the nucleic acids (3), a unit for amplifying the nucleic acids (4), a unit for detecting the nucleic acids (5), a control logic system (6) consisting of a computer of PC type which controls the entire device, pumping means (7), an automatic sampling and distribution means (8) consisting of an automaton, a central control system (9) interfaced with a network (13) which makes it possible to transfer the data from the

control logic system (6) to the central control system (9) consisting of a computer of PC type, a cooling area (10), an inlet and outlet system for the water sample (11) and an area (12) for storing the reagents required for units (1), (2), (3), (4) and (5).

Advantageously, according to the present invention, the sample containing the pathogenic organisms circulates from the unit for concentrating the pathogenic organisms (1) to the inlet of the unit for amplifying the nucleic acids (4), via the units for breaking open the cells (2) and for concentrating the nucleic acids (3), using pumping means (7) advantageously consisting of tubes equipped with valves and/or pumps. Thus, the pumping means (7) form a fluidic system which is able to connect the units (1), (2), (3) and (4). The pumping means (7) according to the invention are activated and controlled by the control logic system (6).

Advantageously, according to the present invention, after the DNA and/or RNA has/have been concentrated, the nucleic acids are manipulated, from the inlet of the unit for amplifying the nucleic acids (4) to the outlet of the unit for detecting the nucleic acids (5), using automatic sampling and distribution means (8) advantageously consisting of an automaton. Thus, the units (4) and (5) are not directly connected to each other (no fluidic system). The automatic sampling and distribution means according to the invention are activated and controlled by the control logic system (6).

Advantageously, according to the present invention, the control logic system (6) consists of at least one computer, advantageously of the PC type, which manages and controls the entire device and which receives, controls and processes all of the analytical data relating to the detection of the pathogenic organisms.

Advantageously, the control logic system (6) according to the present invention is interfaced with a network for transferring, automatically or on demand, the

analytical data relating to the detection of the organisms to a central control system (9).

Advantageously, according to the present invention, the central control system (9) consists of at least one computer. This central control system (9) is able to get and record analytical data, and then to analyse and to control them.

Advantageously, according to the present invention, the device also contains a cooling area (10) for storing the sensitive reagents, preferably a cooling block, which is protected against the condensation of the steam.

Advantageously, according to the present invention, the device is directly connected to the drinking water supply network for automatic sampling. The detection device according to the present invention thus makes it possible to test, in an entirely automated way, samples taken from different supply networks, such as the drinking water network. It can also be reused many times without needing to change the various units used.

Typically, the succession of the various steps of the detection process according to the present invention can be advantageously carried out as follows:

1. The unit (1) for concentrating the pathogenic organisms is flushed, cleaned and then filled with a volume of 100 ml to 5 litres of water, preferably of 2 to 3 litres of water. The water is introduced into the reactor using valves, the opening of which is activated by a computer (6).
2. Various reagents, such as sodium carbonate and calcium acetate, are added to the reactor via valves and rotary pumps, which are controlled by the computer (6). An inclusion precipitate is then formed at the bottom of the chamber, to which the cells and the nucleic acids from the pathogenic organisms adsorb.
3. The supernatant liquid is released, by example by gravity, using a tube and a valve.

4. The precipitate is dissolved using an acid, added to the reactor via a pump which is activated by the computer (6). A buffer agent is also added in the same way.
5. The solution containing the pathogenic organisms is transferred into the unit (2) for breaking open the cells from the organisms, which consists of a bead grinder, by gravity or through the action of a pump controlled by the computer (6). At this stage, the volume of solution entering the bead grinder is from 10 to 100 ml, preferably 50 ml.
6. The cells are broken open by grinding on agitated beads, by means of mechanical energy, for example with a rotary or vibrating shaker or an ultrasound source.
7. The ruptured cells are then transferred, via a pump controlled by the computer (6), into a container made of plastic, of approximately 100 ml.
8. A chaotropic agent and an adsorption-modifying agent are added via valves and pumps which are controlled by the computer (6).
9. The DNA and/or the RNA from the cells is then adsorbed on a solid support, advantageously on microtitration plates containing wells, said wells, themselves, containing an adsorbent material such as a vitreous material. Compressed air may be injected in order to carry the fluid into the wells, and an XY or XYZ frame of reference is used to manipulate the matrix of adsorbent columns. The wells, which are each microcolumns, can thus be manipulated using an automaton activated by the computer (6), the arm of which automaton moves the plate containing the wells according to an XY or XYZ frame of reference.
10. A monovalent complexing agent is then added via valves and pumps controlled by the computer (6), in order to replace the divalent ions complexed with the nucleic acids, with monovalent ions. The nucleic acid/monovalent ion complex thus formed is therefore, at this stage, still attached to the adsorption columns.

11. The nucleic acids are then eluted by adding a buffer, such as TRIS/EDTA, or water using rotary pumps.
12. The nucleic acids are pooled in a tube manipulated, for example, by an automaton. From this stage, all manipulations are carried out using an automatic sampling and distribution means, such as an automaton (8).
13. Optionally, and if it proves to be necessary, the total volume of solution containing the various pooled and eluted nucleic acids is decreased in order to attain volumes of about one hundred or so µl to less than 1 ml, before carrying out the nucleic acid amplification or several amplifications performed in parallel.
14. The nucleic acids are transferred into a thermocycler machine using the automaton (8) and the first amplification is then carried out in the machine by adding, first of all, the reagents for performing the PCR, such as the PCR buffer, the set of primers 1, the TAQ and the nucleotides.
15. The mixture of nucleic acids amplified a first time is then separated into several vials in order to detect a number of organisms at maximum sensitivity with respect to the pathogenic agents whose detection is being sought.
16. A second amplification is then carried out in real time in a second thermocycler machine by adding, in particular, the set of primers 2 (TAQ and nucleotide). Visualization and detection agents, such as fluorescence agents, are also added. The detection agents will thus be able to hybridize to the amplified fragments from the microorganisms. All the sample manipulations (transfers, addition of the various reagents, etc.) are carried out using the automaton (8) mentioned above.
17. The fluorescence is detected in situ during the second amplification (on-line detection).
18. The analytical data relating to the detection of the pathogenic organisms are visualized on the computer (6) and transferred to a central control system (9). All of the

sequential steps of the process are managed and controlled using a control logic system (6) consisting of a computer.

The following examples are given in a nonlimiting capacity and illustrate the present invention.

Examples of implementation of the invention:

Example 1: Precipitation by inclusion of pathogenic agents

First of all, a water sample, taken directly from the drinking water supply network, from the environment or originating from a laboratory or from another source, is received in a 2-litre reactor and is heated to 50°C.

10 ml of 1 M TRIS (buffer agent) are first added so as to attain a pH of 8.5, followed by 25 ml of 1 M calcium acetate. The two compounds are then mixed rapidly, and then the mixing is stopped.

10 ml of 1 M $Na_2CO_3$ are then added and the various compounds are allowed to react for 3 minutes. Mixing is then carried out carefully for approximately 5 seconds, followed by the addition of 10 ml of $Na_2CO_3$, and then reaction medium is allowed to react for a further 3 minutes. Mixing is carried out gently but sufficiently and the reaction medium is again allowed to react for a further 10 minutes. 7.5 ml of 1 M calcium acetate are added and allowed to react for 3 minutes. Mixing is carried out carefully, 10 ml of $Na_2CO_3$ are added and the mixture is again allowed to react for 5 minutes.

The supernatant is decanted by opening a valve. The precipitate is dissolved by adding 13 ml of 20% formic acid. The mixture is then buffered by adding 2 M TRIS base to increase the pH to 7.2.

Example 2: Amplification by PCR and detection

2.1 A thermocycler machine with integrated on-line detection, such as the LightCycler machine sold by Roche Molecular Biochemicals, is used to detect the amplification of the specific DNA. 32 samples are manipulated in

parallel, and 40 amplification cycles are carried out at high temperature (from 90°C to 100°C), the 40 cycles lasting approximately 45 minutes. The amplification and the detection take place in capillary tubes. Detection limit is about one pathogenic organism.

2.2 As an alternative, it is also possible to use the ABI prism 7700 detection system, sold by PE Biosystems, processing 96 samples in parallel (two hours), over 40 cycles for the detection of more than 10 samples in a vial, for approximately 2.5 hours.

Sensitivities of the order of a few femtogrammes of total DNA/RNA (which is equivalent to one single cell) are thus obtained. Detection limit is about one pathogenic organism.

CLAIMS

1. Process for detecting the presence of pathogenic organisms in a water sample, comprising the succession of
5 the following steps:
concentration of the pathogenic organisms,
breaking open of the cells of the pathogenic organisms,
concentration of DNA and/or of RNA,
at least one nucleic acid amplification, and
10 a qualitative and/or quantitative detection of DNA and/or RNA representative of the water contamination by said pathogenic organisms;
characterized in that all of the sequential steps of the process are controlled fully automatically using a control
15 logic system.

2. Detection process according to Claim 1, characterized in that the pathogenic organisms detected are bacteria, viruses, fungi and/or protozoa.

3. Detection process according to Claim 1 or 2,
20 characterized in that the starting volume of the water sample is of more than 50 milliters.

4. Detection process according to any one of Claims 1 to 3, characterized in that the nucleic acid amplification is carried out by a real-time polymerase chain reaction.

25 5. Detection process according to any one of Claims 1 to 4, characterized in that the concentrating of the pathogenic organisms is carried out by precipitation, coprecipitation or precipitation by inclusion.

6. Detection process according to Claim 5,
30 characterized in that the concentrating is carried out in the presence of divalent metal and carbonate ions advantageously chosen from the group consisting of calcium carbonate, strontium carbonate and barium carbonate.

7. Detection process according to any one of Claims 1
35 to 6, characterized in that the breaking open of the cells is carried out by grinding on agitated beads.

8. Detection process according to Claim 7, characterized in that the beads are agitated by ultrasound, by mechanical shaking or by mechanical vibration.

9. Detection process according to any one of the preceding claims, characterized in that, during the breaking open, or subsequent to the breaking open of the cells and prior to the concentrating of DNA and/or of RNA, at least one chaotropic agent, advantageously a guanidine salt, such as guanidine thiocyanate, and at least one adsorption-modifying agent, advantageously an alcohol such as ethanol, are added to the reaction medium containing the cells.

10. Detection process according to any one of the preceding claims, characterized in that the concentrating of DNA and/or of RNA is carried out by reversible adsorption of the nucleic acids onto a solid support containing an adsorbent material, followed by elution of these acids.

11. Detection process according to Claim 10, characterized in that the elution of the nucleic acids is carried out by washing the absorbent material using at least one solvent containing monovalent ions, advantageously chosen from the group consisting of sodium thiocyanate, potassium thiocyanate and EDTA.

12. Detection process according to any one of the preceding claims, characterized in that the detection of the pathogenic organisms is an on-line detection, advantageously carried out by fluorescence, by luminescence or by mass spectrometry.

13. Detection process according to any one of the preceding claims, characterized in that it also comprises a prior filtration step.

14. Detection process according to any one of the preceding claims, characterized in that it also comprises a prior step for treating the water sample by heat shock and/or by a nutrition stress and/or by adding a gene-inducing chemical compound in order to induce the RNA of

specific genes and to detect the viable pathogenic organisms in said water sample.

15. Detection process according to Claim 14, characterized in that the heat shock is carried out by heat pulse for less than 3 hours.

16. Detection process according to Claim 14 or 15, characterized in that the gene-inducing chemical compound is lactose or IPTG so as to induce the lac Z RNA.

17. Detection process according to any one of the preceding claims, characterized in that the control logic system consists of a computer.

18. Detection process according to any one of the preceding claims, characterized in that the analytical data relating to the detection of the organisms are automatically transferred to a central control system.

19. Detection process according to any one of the preceding claims, characterized in that the process can function continuously and autonomously for a period of time greater than or equal to one day, preferably from three days to seven days.

20. Device for detecting the presence of pathogenic organisms in a water sample, comprising:
a unit for concentrating the pathogenic organisms (1),
a unit for breaking open cells from the pathogenic organisms (2),
a unit for concentrating DNA and/or RNA (3),
a unit for amplifying nucleic acids (4), and
a unit for qualitatively and/or quantitatively detecting DNA and/or RNA (5) representative of the water contamination by said pathogenic organisms;
characterized in that it also comprises:
at least one a control logic system (6) which controls fully automatically the sequential treatment steps carried out by units (1) to (5),
pumping means (7),
automatic sampling and distribution means (8), and
a central control system (9).

21. Detection device according to Claim 20, characterized in that the unit for amplifying the nucleic acids (4) consists of at least one thermocycler polymerase chain reaction machine.

5 22. Detection device according to Claim 20 or 21, characterized in that the unit for concentrating the pathogenic organisms (1) is a reactor made of metal, comprising a system for introducing the water sample, a system for introducing various reagents, an agitation

10 system, a system for evacuating the supernatant liquid, a system for evacuating the precipitate, advantageously placed at the bottom of the reactor, and a heating system advantageously consisting of a heating jacket placed around the reactor.

15 23. Detection device according to any one of Claims 20 to 22, characterized in that the unit for breaking open the cells (2) contains a bead grinder.

24. Detection device according to any one of Claims 20 to 23, characterized in that the unit for concentrating DNA

20 and/or RNA (3) comprises one or more solid support(s) containing a reversible adsorption material for nucleic acids, advantageously a vitreous material.

25. Detection device according to Claim 24, characterized in that the solid supports consist of

25 microtitration plates containing multiwells, advantageously 60-, 96- or 384-well plates.

26. Detection device according to Claim 25, characterized in that the wells of the microtitration plates are used only once and are moved automatically in

30 order to receive the liquid sample originating from the unit for breaking open the cells from the organisms.

27. Detection device according to Claim 26, characterized in that the automatic movement of the wells is carried out using an automaton.

35 28. Detection device according to any one of Claims 20 to 27, characterized in that the detection unit (5)

consists of a fluorescence detector, advantageously comprising an electronic camera.

29. Detection device according to any one of Claims 20 to 28, characterized in that the sample containing the pathogenic organisms circulates from the unit for concentrating the pathogenic organisms (1) to the inlet of the unit for amplifying the nucleic acids (4), via the units for breaking open the cells (2) and for concentrating the nucleic acids (3), using pumping means (7) advantageously consisting of tubes equipped with valves and/or pumps.

30. Detection device according to any one of Claims 20 to 29, characterized in that after the DNA and/or RNA has/have been concentrated, the nucleic acids are manipulated, from the inlet of the unit for amplifying the nucleic acids (4) to the outlet of the unit for detecting the nucleic acids (5), using automatic sampling and distribution means (8) advantageously consisting of an automaton.

31. Detection device according to any one of Claims 20 to 30, characterized in that the control logic system (6) consists of at least one computer of the PC type which controls the entire device.

32. Detection device according to any one of Claims 20 to 31, characterized in that the control logic system (6) is interfaced with a network for transferring, automatically or on demand, the analytical data relating to the detection of the organisms to a central control system (9).

33. Detection device according to any one of Claims 20 to 32, characterized in that the central control system (9) consists of at least one computer.

34. Detection device according to any one of Claims 20 to 33, characterized in that the device also contains a cooling area (10) for storing the sensitive reagents, preferably a cooling block.

35. Detection device according to any one of Claims 20 to 34, characterized in that the device is directly connected to the drinking water supply network for automatic sampling

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