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(54) **Title:** FORMULATIONS OF MICROORGANISM COMPRISING PARTICLES AND USES OF SAME

(57) **Abstract:** Methods of reducing nitrate overload in water, of purifying food industry wastewater or of purifying pharmaceutical wastewater are disclosed. The methods comprising contacting the water or wastewater with a particle comprising: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water. Methods of treating water, of purifying municipal wastewater, of purifying food industry wastewater, of purifying pharmaceutical wastewater, of reducing sludge production and stabilizing a wastewater purification treatment are also disclosed.



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FORMULATIONS OF MICROORGANISM COMPRISING PARTICLES AND USES  
OF SAMEFIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to microorganism-comprising particles and, more particularly, but not exclusively, to the use of same for the removal of contaminants from water or soil, facilitating de-nitrification, for treatment of diseases and for the production of pharmaceutical and cosmetic compositions.

Water quality management is one of the world's most significant concerns. As industry becomes more complex and advanced, problems associated with water pollution become more significant. Consequently, advanced wastewater treatment technology is required. Concentration of industrial waste nutrients such as heavy metals, phosphorous, phenols and oils are difficult to reduce to safe environmental levels. Increasing environmental awareness and the toughening governmental policies, demand new environmentally friendly ways to clean up contaminants using low cost methods and materials. These new technologies for removing nutrients from large volume of wastewater must be economically feasible. For example, physicochemical procedures, such as chemical precipitation, utilizing flocculation- coagulation-sedimentation processes and ion exchange adsorption to exclude heavy metals from wastewater are currently used.

Biological materials and methods, have been extensively studied, and answer some of the above demands, being both economically feasible and capable of coping with large volumes of wastewater.

Biosorption (the ability of certain types of inactive, dead, microbial biomass to bind and concentrate heavy metals from even very diluted aqueous solutions) has proven to be an excellent way to treat industrial waste effluents, offering significant advantages such as low cost, availability, efficiency and ease of operation. Biosorption from aqueous effluents has become a potential alternative to the existing technologies of removal hazard nutrients from industrial wastewater [Shuttleworth, K.L. and R.F. Appl Environ Microbiol (1993). 59(5): 1274-1282].

Bioaccumulation, the gradual accumulation of a certain chemical into living organisms, has been used to clean up contaminated environments such as copper-, zinc- and nickel-contaminated wastewater [Kara Y., *Int. J. Environ. Sci. Tech.* (2005) 2(1): 63-67].

Biodegradation, the process by which live microorganisms are capable of removing contaminants (e.g. nitrates) from organic material has also been extensively used to clean up contaminated environments (e.g. wastewater). Live microorganisms have the naturally occurring, microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons (e.g. oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pharmaceutical substances, radionuclides and metals.

Microorganisms have the ability to remove contaminants (e.g. heavy metals, phosphates and oils) from wastewater by degradation or absorption and the efficiency of such biological processes is high, estimated to give a yield of exclusion of over 99 %. As such, a high percentage of ongoing academic research is focused on identifying specialized microorganisms (e.g. bacteria, yeast, fungi and algae) and adapting them to hostile conditions such as wastewater environment. The main challenge is to use living microorganisms in unstable conditions (e.g. pH variations, nutrients inhibition, nutrient enhancement, etc.).

Furthermore, wastewater flora consists of various microorganisms populations co-existing in a steady state. The efficient use of microorganisms in wastewater treatment requires that the introduced culture be genetically stable and would integrate along with the wastewater natural flora. Introduction of the new culture may be problematic as it may interrupt the flora stability and may lead to undesired effects such as an undesired withdrawal to the former steady state or to elimination of the new microorganisms. Thus, efficiency of the biological process or treatment depends on the threshold concentration (biomass) of the introduced culture. Since the introduced microorganism culture is challenged by natural selection forces (due to environmental adaption), reaching the necessary biomass may be impossible and survival of the introduced culture is extremely difficult.

Biosorption and biodegradation processes using selected bacteria to exclude contaminant nutrients have been commercially previously described. For example,

BioPetroClean (BPC) utilizes a bacterial cocktail to remove both dissolved and emulsified hydrocarbons from water, soil, oil storage and transportation tanks. Their technology combines a unique mixture of naturally-occurring bacteria that feed on petroleum hydrocarbons combined with a supplemental nutrient-mix and a controlled oxygen tension and pH which ensures optimal bio-degradation. The BPC technology is based on adaptation of planktonic bacteria blends with the ability to degrade petroleum hydrocarbons.

Furthermore, bioprocessors have frequently been used to grow useful cells or to clean contaminated effluent, such as water. More specifically, biofilms have been widely used because an active biomass produced in the reactor allows large volumetric loadings and good effluent quality without the need for separation of solids. The biofilm bioreactors have been generally categorized as continuously stirred tank reactors (CSTRs), fixed-bed and fluidized bed (described in detail in U.S. Pat. No. 6,235,196).

Numerous publications have described the use of microorganisms to exclude contaminant nutrients such as heavy metals, phosphates and oils from wastewater. Following are some of the cited art.

U.S. Pat. No. 4,530,763 describes methods for treating waste fluids to remove selected chemicals (e.g. minerals and metals) using bacterial cultures. According to their teachings, the bacterial culture is first transferred to a nutrient medium to enable satisfactory bacterial cell growth. The bacterial cells are then attached to a porous fiber webbing supported in a suitable container, the nutrient medium is then replaced with waste fluid for a period of time sufficient to attach the chemical to the bacterial cells. The waste fluid is then removed from the container and the chemical separated from the fiber webbing.

U.S. Pat. No. 6,423,229 describes bioreactor systems for biological nutrient removal. Specifically, U.S. Pat. No. 6,423,229 teaches an integrated biological treatment process and bioreactor system which provides means for simultaneous removal of biodegradable solids, nitrogen and phosphate from water and wastewater. The system comprises microbial consortia immobilized in separate bioreactors for anaerobic processes, phosphate removal and denitrification.

## SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of reducing nitrate overload in water, the method comprising contacting the water with a particle comprising (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms selected capable of facilitating denitrification of the water; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water, thereby reducing nitrate overload in water.

According to an aspect of some embodiments of the present invention there is provided a method of purifying food industry wastewater, the method comprising contacting the wastewater with a particle comprising: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water, thereby purifying the food industry wastewater.

According to an aspect of some embodiments of the present invention there is provided a method of purifying pharmaceutical wastewater, the method comprising contacting the wastewater with a particle comprising: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water, thereby purifying the pharmaceutical wastewater.

According to an aspect of some embodiments of the present invention there is provided a method of purifying municipal wastewater, the method comprising contacting the municipal wastewater with a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater and a second population of dried microorganisms selected for

purification of petroleum wastewater, under conditions that allow the microorganisms to decontaminate the municipal wastewater, thereby purifying the municipal wastewater.

According to an aspect of some embodiments of the present invention there is provided a method of purifying food industry wastewater, the method comprising contacting the food industry wastewater with a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of food industry wastewater and a second population of dried microorganisms selected for purification of municipal wastewater, under conditions that allow the microorganisms to decontaminate the food industry wastewater, thereby purifying the food industry wastewater.

According to an aspect of some embodiments of the present invention there is provided a method of purifying pharmaceutical wastewater, the method comprising contacting the pharmaceutical wastewater with a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of pharmaceutical wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater, under conditions that allow the microorganisms to decontaminate the pharmaceutical wastewater, thereby purifying the pharmaceutical wastewater.

According to an aspect of some embodiments of the present invention there is provided a method of treating water, the method comprising contacting the water with a plurality of particles, wherein the plurality of particles comprise: (i) a first population of dried microorganisms selected for purification of municipal wastewater; (ii) a second population of dried microorganisms selected for purification of petroleum wastewater; and (iii) a third population of dried microorganisms selected for de-nitrification of water; under conditions that allow the microorganisms to purify the water, thereby treating the water.

According to an aspect of some embodiments of the present invention there is provided an article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater.

According to an aspect of some embodiments of the present invention there is provided an article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of food industry wastewater and a second population of dried microorganisms selected for purification of municipal wastewater.

According to an aspect of some embodiments of the present invention there is provided an article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of pharmaceutical wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater.

According to an aspect of some embodiments of the present invention there is provided an article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater, a second population of dried microorganisms selected for purification of petroleum wastewater and a third population of dried microorganisms selected for de-nitrification of wastewater.

According to an aspect of some embodiments of the present invention there is provided an article of manufacture comprising a plurality of particles, wherein the plurality of particles comprises a population of dried microorganisms selected for de-nitrification of wastewater.

According to an aspect of some embodiments of the present invention there is provided a method of reducing sludge production during wastewater purification, the method comprising contacting the wastewater with a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater, under conditions that allow the microorganisms to decontaminate the wastewater, thereby reducing sludge production during the wastewater purification.

According to an aspect of some embodiments of the present invention there is provided a method of stabilizing a wastewater purification treatment, the method comprising contacting the wastewater with a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification

of municipal wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater, under conditions that allow the microorganisms to decontaminate the wastewater, thereby stabilizing the wastewater purification treatment.

According to an aspect of some embodiments of the present invention there is provided a particle suitable for de-nitrification, comprising: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms, wherein the dried microorganisms are selected for facilitating de-nitrification; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water.

According to an aspect of some embodiments of the present invention there is provided a particle suitable for purification of municipal wastewater, comprising: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms, wherein the dried microorganisms are selected for purification of municipal wastewater; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water.

According to an aspect of some embodiments of the present invention there is provided a particle suitable for purification of food industry wastewater, comprising: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms, wherein the dried microorganisms are selected for purification of food industry wastewater; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water.

According to an aspect of some embodiments of the present invention there is provided a particle suitable for purification of pharmaceutical wastewater, comprising: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms,

wherein the dried microorganisms are selected for purification of pharmaceutical wastewater; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water.

According to an aspect of some embodiments of the present invention there is provided a particle suitable for oxygen enrichment, comprising: (i) at least one inner core which comprises a solid oxygen release compound; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water.

According to some embodiments of the invention, the water is wastewater.

According to some embodiments of the invention, the wastewater is selected from the group consisting of petroleum wastewater, municipal wastewater, pharmaceutical wastewater, nitrogen enriched wastewater and food industry wastewater.

According to some embodiments of the invention, the wastewater comprises municipal wastewater.

According to some embodiments of the invention, the contacting is effected under anaerobic conditions.

According to some embodiments of the invention, the microorganisms are selected from the group consisting of *Alcaligenes*, *Pseudomonas*, *Methylobacterium*, *Bacillus*, *Paracoccus* and *Hyphomicrobium* and a combination of same.

According to some embodiments of the invention, the contacting is effected in the absence of non-particle associated organic matter.

According to some embodiments of the invention, contacting with (i) and (ii) are effected simultaneously or sequentially.

According to some embodiments of the invention, contacting with (i) and (ii) are effected under aerobic conditions.

According to some embodiments of the invention, contacting with (iii) is effected under anaerobic conditions

According to some embodiments of the invention, contacting with (iii) is effected following (i) and (ii).

According to some embodiments of the invention, the method further comprises at least one particle suitable for oxygen enrichment.

According to some embodiments of the invention, contacting with (i) and (ii) and the particles suitable for oxygen enrichment are effected simultaneously.

According to some embodiments of the invention, the aerobic conditions comprise the presence of at least one particle suitable for oxygen enrichment.

According to some embodiments of the invention, the wastewater is nitrogen enriched wastewater.

According to some embodiments of the invention, the article of manufacture further comprises at least one particle suitable for oxygen enrichment.

According to some embodiments of the invention, the plurality of particles comprise at least two non-identical particles.

According to some embodiments of the invention, the ratio of the first population of dried microorganisms and the second population of dried microorganisms is selected from the group consisting of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 and 10:1.

According to some embodiments of the invention, the ratio of the first population of dried microorganisms and the second population of dried microorganisms is 6:1.

According to some embodiments of the invention, the ratio of the first population of dried microorganisms and the second population of dried microorganisms is 1:1.

According to some embodiments of the invention, the ratio of the first population of dried microorganisms, the second population of dried microorganisms and the third population of dried microorganisms is selected from the group consisting of 1:1:1, 1:1:2, 1:1:3, 1:1:4, 1:1:5, 1:1:6, 1:1:7, 1:1:8, 1:1:9 and 1:1:10.

According to some embodiments of the invention, the ratio of the first population of dried microorganisms, the second population of dried microorganisms and the third population of dried microorganisms is 1:1:2.

According to some embodiments of the invention, the plurality of particles is selected such that the ratio of the particles to the wastewater is between about 0.01 to 5 particles per cube wastewater per day.

According to some embodiments of the invention, each of the particles comprise: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and the dried microorganisms; and (iii) an outer

porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water.

According to some embodiments of the invention, the particles are pre-activated in a liquid prior to the contacting.

According to some embodiments of the invention, the particles are pre-activated for about 24 to 96 hours prior to the contacting.

According to some embodiments of the invention, contacting is effected for a period of about 2 weeks to about 45 weeks.

According to some embodiments of the invention, the solid matrix of nutrients comprises an agar.

According to some embodiments of the invention, the microorganisms comprise bacteria.

According to some embodiments of the invention, the bacteria comprise freeze-dried bacteria.

According to some embodiments of the invention, the microorganisms comprise yeast.

According to some embodiments of the invention, the inner core is coated with a control release polymer.

According to some embodiments of the invention, the inner membrane or the inner core further comprise an enzyme.

According to some embodiments of the invention, the inner core further comprises an agent selected from the group consisting of an amino acid, an enzyme, a peptide, a protein, a carbohydrate, a sugar, an iron, a salt and an essential element.

According to some embodiments of the invention, the inner core is devoid of at least one agent selected from the group consisting of an amino acid, a peptide, a protein, a carbohydrate, a sugar, an iron, a salt and an essential element.

According to some embodiments of the invention, the particles support biofilm formation within.

According to some embodiments of the invention, the inner membrane further comprises additional elements which support biofilm formation thereon.

According to some embodiments of the invention, the additional elements comprise glass beads.

According to some embodiments of the invention, the inner membrane further comprises activated carbon granules or activated carbon chips within.

According to some embodiments of the invention, the inner membrane further comprises an oxygen release compound within.

According to some embodiments of the invention, the water-soluble polymer comprises gelatin.

According to some embodiments of the invention, the gelatin is further coated with a control release polymer.

According to some embodiments of the invention, the outer porous membrane is fabricated from a polymer selected from the group consisting of PVAL (polyvinyl-alcohol), Polyethersulfone (PES), Cellulose Acetate, Cellulose Nitrate, Ethyl Cellulose, Nitrocellulose Mixed Esters, Polycarbonate film, Nylon, PVDF(poly(vinylidene fluoride)) and Polysulfone.

According to some embodiments of the invention, the outer porous membrane is fabricated from a polymer comprising Cellulose Acetate.

According to some embodiments of the invention, the porous membrane is resistant to biofilm formation.

According to some embodiments of the invention, the pore of the porous membrane is less than 0.85  $\mu\text{m}$ .

According to some embodiments of the invention, the particle is between 0.5-30  $\mu\text{m}$  in length.

According to some embodiments of the invention, the particle is devoid of microorganisms.

According to some embodiments of the invention, the particle comprises microorganisms surrounded by the inner membrane.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

## BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1A is an illustration of the components of the particle in an active state. The particle components comprise (1) an outer semi-permeable membrane (for nanofiltration/microfiltration), (2) a nutrient-comprising inner core, (3) dried microorganisms, (4) optionally, glass/polymer beads and other components such as active carbon (not shown herein) may be added therein.

FIG. 1B is a photograph depicting a particle prototype with a cellulose acetate membrane.

FIG. 2 is a graph depicting viability (log 10) of freeze-dried *E. coli*. bacteria. Of note, the bacteria viability levels were kept stable for at least 5 weeks after rehydration at an average of 9.5 CFU/ml. The recovery rate of the culture was 1.6 %-1.8 %.

FIG. 3 is a photograph depicting a freeze-dried *E. coli* TG1 culture.

FIGs. 4A-B are photographs depicting the inner core of the particle. Figure 4A depicts the inner core after polymerization (left) and in comparison to a dried core (right). Figure 4B depicts a lateral view of an inner core after polymerization and storage of inner cores in a vial (sterilized by U.V radiation).

FIG. 5 is photograph depicting water soluble gelatin capsules which contain all of the inner components.

FIG. 6 is a photograph of ethyl cellulose coated particles. Each particle was coated with 15 ml or 13 ml of the polymer solution.

FIGs. 7A-B are photographs depicting particles coated with ethyl cellulose 8 % or cellulose acetate 8 % (in a time slides of weeks). Figure 7A depict ethyl cellulose coated particles. The right particle is an empty gelatin capsule and the other particles are 1-4 weeks old ethyl cellulose coated particles. Figure 7B depicts cellulose acetate coated

capsules. The right particle is an empty gelatin capsule and the other particles are 1-3 weeks old cellulose acetate particles.

FIG. 8 is a graph depicting water penetration flow rate (% of total particle maximum weight after injecting water into the particles). Two types of 8 % Ethyl cellulose (EC) coated particles were examined: one particle was coated with 15 ml and the other with 13 ml of the polymer solution (1.2 gr or 1.04 gr of ethyl cellulose and castor oil). The weights of the particles were 0.64 gr (15 ml coated particle) and 0.41 gr (13 ml coated particle). Particle water content of 30 % was the point of particle activation. In the 8 % Ethyl cellulose membrane particles, this was achieved after 72 h or 96 h for 15 ml (black circles) and 13 ml (black diamonds) coated particles, respectively. The particles that were coated with 15 ml of the polymeric solution reached maximum weight while the particles that were coated with 13 ml of the polymeric solution reached only 87 % of the maximum particle weight. Particles which did not contain bacteria (black triangles) displayed a significant inhibition of water flow into the particle.

FIG. 9 is a graph depicting water flow rate into particles coated with 8 % cellulose acetate and comprising different types of bacteria (*E.coli* TG1 and *E.coli* TG1 pChv1). The bacteria type had no influence on the water flow rate into the particles.

FIG. 10 is a graph depicting water flow rate into particles coated with either ethyl cellulose or cellulose acetate. The cellulose acetate particles displayed a faster activation point compared to the ethyl cellulose particles.

FIGs. 11A-C are photographs depicting activation and biocompatibility test systems. Each of the bottles or flasks used for testing contained saline and several particles (comprising different outer membrane coatings). The particles were maintained in the test systems for 5 weeks and viability was checked on a weekly basis. Figure 11A shows flasks which contained 8 % ethyl cellulose coated particles (13 ml); Figure 11B shows a bottle which contained 8 % ethyl cellulose coated particles (15 ml); and Figure 11C shows a bottle which contained 8 % cellulose acetate coated particles (8 ml).

FIG. 12 is graph depicting bacteria viability ( $\log_{10}$  CFU/ml) within the activated cellulose acetate membrane particle. Liquid phase viability counts were carried out on a weekly basis. First, the liquid was pumped by using a syringe and a needle, next the obtained sample was serial diluted with saline. Two types of particles were tested: one

which contained an E.coli TG1 culture and the other which contained E.coli TG1 pChv1. The outer membrane of both particles was identical and contained cellulose acetate 8 % (each particle was sprayed with 8 ml of the polymer solution). Of note, the viability counts obtained from the two particles were similar. Furthermore, after 3 weeks of particle activation, a stable bacteria viability number was established (at an average of 8 log<sub>10</sub> CFU/ml) and after 5 weeks of particle activation, the bacteria concentration within the particle was high. The results represent bacteria viability of one representative particle (the bar represents the viability counts of each representative particle).

FIG. 13 is a graph depicting bacteria viability (log<sub>10</sub> CFU/ml) within the activated ethyl cellulose membrane particle. Liquid phase viability counts were carried out on a weekly basis. First, the liquid was pumped by using a syringe and a needle, next the obtained sample was serially diluted with saline. The outer membrane of the particle was coated with ethyl cellulose 8 % (each particle was sprayed with 15 ml of the polymer solution). Particle biocompatibility was tested on a weekly basis for up to 4 weeks. Of note, a typical growth curve was observed. Thus, in the first week an environment culture adaptation of the bacterial culture was observed which was followed by logarithmic phase for more than 1 week. After 4 weeks, the culture concentration was 10,000,000 bacteria per 1 ml liquid.

FIG. 14 is a diagram depicting the inducer homo-serine lactone. Homo-serine lactone was used as a model for molecule trafficking across the particle membrane.

FIG. 15 is a photograph depicting molecule trafficking across the particle membrane (a validation experiment). The membrane permeability validation system included two systems: 1) the experimental system in which the inducer (1 µg/ml) was added to the particle medium (saline), and 2) the control system which did not contain the inducer in the particle medium. Each system contained one particle within a gently vortexed saline medium and in both systems the particles contained the same bacterial culture (E. coli TG1 pchv1, that was harboring the luciferase system). The particles were incubated inside each of the mediums for one hour prior to extraction of the particle inner medium (containing the planktonic bacterial culture) using a syringe with a needle. The sample collected was read in a Bio-Tek spectrophotometer (light detector sensitivity 125) on 96 wells plate. Light emission was observed and compared between the test

culture, the control system and a blank medium (saline). The ratio between the results represents the intensity of the inducer transport.

FIG. 16A is a diagram of the experimental system of petroleum wastewater (hydrocarbon biodegradation), depicted herein as NatiCap<sup>TM</sup> petroleum treatment.

FIGs. 16B-C are photographs depicting the test model. Figure 16B shows a side view and Figure 16C shows an upper view of the test system which includes the biological reactor, NatiCaps<sup>TM</sup> (75 particles), diffusers, samples valve and the air pump.

FIG. 17 is an illustration a typical a process flow draw (PFD) of heavy metals wastewater treatment (physicochemical technology in use for decontamination of petroleum wastewater and for heavy metals containing wastewater).

FIG. 18 is an illustration of a typical a process of refinery wastewater treatment (SWS - Sour Water Stripper).

FIGs. 19A-B are illustrations of two methods of growing non-identical particles containing different microorganisms. Figure 19A depicts culturing several non-identical particles in one host reactor. Figure 19B depicts culturing the different non-identical particles in a host reactor which has internal chambers (separated by a perforated separator). The perforated separator pore size is selected smaller then the particle size. Each internal chamber contains one type of particles. The feeding liquid is circulated between the reactor chambers.

FIG. 20 is an outline design of the bioreactor and sedimentation tanks.

FIGs. 21A-B depict a process flow diagram (Figure 21A) and provides a photograph of the on-site test system and control system (Figure 21B). Both systems comprise a sedimentation tank and a bioreactor, however, the bioreactor of the test system includes the particles of the present invention (municipal and petroleum particles as described in detail in Examples 5-6 below). Of note, the influent flow into each of the bioreactors was posionted above the bioreactor rather than into the upper third of the bioreactor.

FIGs. 22A-B are line graphs depicting the reduction in organic load in both the control and test bioreactors. The y-axes represent the ratio (%) of the residual organic load inside each of the bioreactors:  $1 - (\text{Effluents/Influents}) \times 100$ . The x-axes represent the days following particle activation (i.e. the start point of the trial).

FIGs. 23A-L are photographs depicting two different sludge sedimentation tests. In the first sludge sedimentation test (Figures 23A-F), the total volume of the sludge in the test and control bioreactors was 30 ml (15 % of total volume) and 25 ml (12.5 % of total volume), respectively, after 30 minutes of incubation. Thus, the difference between both bioreactors was 2.5 % (5 ml). In the second sedimentation test (10 days later, Figures 23G-L), the total volume of the sludge in the test and control bioreactors was 20 ml (10 % of total volume) and 45 ml (22.5 % of total volume), respectively, after 30 minutes of incubation. Thus, the test bioreactor had significantly less sludge (12.5 %, 25 ml reduction).

FIGs. 24A-D are photographs depicting foaming & overflow marks on the control bioreactor (Figures 24B and 24D) and on the particle comprising test bioreactor (Figures 24A and 24C).

FIGs. 25A-B are photographs depicting the 35 particles of the present invention after they were pulled out from the test bioreactor (Figure 25A) and characterized (Figure 25B). Of note, the inventor did not observe any broken or defected particles. The particles were further taken for bacteriological analysis and were then destroyed using 10 % chloride solution.

FIGs. 26A-C depict the outline design diagram of the pilot (Figure 26A) and provides photographs of the on-site test system (Figure 26B) and of the cartridge (particles housing) which was integrated within the cap of the bioreactor (Figure 26C). Of note, the particles were introduced into the bioreactor via the particles housing (cartridge) which was poisoned in the middle of the bioreactor, above the ring diffuser.

FIGs. 27A-D are line graphs depicting the chemical analysis results of the influents and effluents of the food water industry wastewater (whey wastewater) over time (days).

FIGs. 28A-D are line graphs depicting chemical analysis of the test bioreactor comprising the particles (pound B) and the control bioreactor (pound A). Figure 28A illustrates chemical oxygen demand (COD) concentrations; Figure 28B illustrates biological oxygen demand (BOD) concentrations; Figure 28C illustrates Mixed Liquor Suspended Solids (MLSS) concentrations; and Figure 28D illustrates total suspended solids (TSS) concentrations.

FIGs. 29A-C are photographs illustrating introduction of the particles using cartridges. The cartridges contained up to 2000 particles each coated with 1 mm pores size mesh.

FIGs. 30A-C are photographs illustrating the pilot system and particle introduction for total nitrogen reduction in purified wastewater. Inflow was provided from municipal wastewater treatment plant (MWWTP) effluents. The pilot system was established inside a structure (with no projection of direct sun light). The pilot system's total volume was 2 m<sup>3</sup> and had 3 chambers: 2 aeration chambers (including diffusers, vortex and pH meter) and one sedimentation chamber. The inflow from MWWTP was introduced to the first aerated & circulated chamber at rate flow of 1 L/minute (HRT-24 h of the nitrification process, and HRT-12 hours of de-nitrification process), and was transferred to the second aerated chamber. After the nitrification process, the effluents were transferred to the anoxic sedimentation chamber for the de-nitrification process. The particles were packed within a mesh pouch which was connected to a metal weight (approximately 1 kg). 100 nitrification particles were introduced to the first aerated & circulated chamber. Additional 100 de-nitrification particles were introduced to the sedimentation chamber.

FIGs. 31A-C are line graphs illustrating chemical analysis results of Ammonia, Total Kjeldahl Nitrogen, total Nitrogen of the influent (inflow) and effluent (outflow). The last point of the analysis was preformed a few days after removal of the particles from the test system.

FIGs. 32A-B are line graphs illustrating chemical analysis results of Nitrate and COD of the influent (inflow) and effluent (outflow). During the second stage of the test (after 50 days), the de-nitrification process started to engage, resulting in nitrite concentration reduction. The last point of the analysis was preformed a few days after the removal of the particles from the test system.

## DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to microorganism-comprising particles and, more particularly, but not exclusively, to the use of same for the removal of contaminants from water or soil, facilitating de-nitrification, for treatment of diseases and for the production of pharmaceutical and cosmetic compositions.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

While reducing the present invention to practice, the present inventor has generated novel particles which comprise microorganisms within. The particles comprise an outer porous membrane which is selected such that it allows trafficking of molecules of a particular size (e.g. water molecules or proteins, carbohydrates, lipids) while inhibiting trafficking of molecules of a larger size (e.g. microorganisms). The particles further comprise an inner core which supports microorganism growth and prosperity.

The present inventor demonstrated the use of the particles for wastewater treatment. Particles were generated which contained bacteria, inner cores, activated Carbon and degradation enzymes. These particles were placed in petroleum wastewater and were shown to significantly increase BOD (biological oxygen demand) and TSS (total suspended solids) levels (in both aerobic and anoxic stages) and to significantly decrease COD (chemical oxygen demand) levels (in the aerobic stages) indicating hydrocarbon degradation. Moreover, the particles exhibited good biocompatibility after 3 weeks within the petroleum wastewater and the necessary biomass within the particles developed within a short period (within 3 days). The present teachings portray the use of the particles for removal of contaminants, such as from waste water or soil.

The present inventor further demonstrated, using a luciferase test system, that molecules of certain size can be transported back and forth through the particle outer membrane. More specifically, the present inventor generated particles containing genetically transformed bacteria (i.e. comprising a plasmid that contained the entire luciferase system). The system inducer (i.e. Homo serine lactone - C10 hydrocarbon), was added to the medium in which the particle was placed and upon penetration of the particle (and contact with the bacteria), transcription of the plasmid was induced. These results indicate that the particles comprise pores of a size which allow the passage of the inducer from the medium into the particle.

The present inventor has further shown purification of municipal wastewater, petroleum wastewater, food industry wastewater and pharmaceutical wastewater using the novel particles. Specifically, the present inventor has shown in an on-site wastewater treatment facility that municipal/petroleum wastewater can be efficiently and stability treated using a combination of particles comprising bacteria formulated for petroleum wastewater treatment and for municipal wastewater treatment (e.g. at a ratio of 1:6, see Examples 5 and 6, hereinbelow). The use of this combination of particles led to a stable purification process (see Figures 22A-B and 24A-D) for a prolonged period of time (e.g. for 9 months, see Example 9 in the Examples section which follows) and to reduced sludge production within the sedimentation tank. Moreover, the present inventor has shown in an on-site wastewater treatment facility that food industry wastewater (e.g. whey wastewater) can be efficiently and stability treated using a combination of particles comprising bacteria formulated for food industry wastewater treatment and for municipal wastewater treatment (e.g. at a ratio of 1:6, see Example 7, hereinbelow). Moreover, the present inventor has successfully utilized the particles of the present invention for de-nitrification of wastewater. Specifically, it was shown in Example 10 of the Examples section which follows, that ammonia and nitrate can be efficiently removed from water (e.g. treated wastewater) without the addition of non-particle comprising organic matter. Taken together, the present teachings suggest the use of the particles for wastewater treatment and purification.

The present teachings provide for the first time means of stabilizing a biological process in a wastewater facility by:

A. Developing an additional biological process i.e. the activated sludge of the bioreactor and the biomass inside the particles. Thus, instead of just one biological process, the present teachings enable co-activation of two biological processes.

B. Removal of anti-microbial agents from the wastewater using the present teachings enables protection of the main biological process (i.e. the bioreactor sludge) and keeps a high yield of the biodegradation process.

The present teachings further suggest the use of the particles for production of desired molecules (e.g. biopolymers including polypeptides, polysaccharides etc.). Such molecules may be used in pharmaceutical or cosmetic compositions. Genetically transformed microorganisms (e.g. bacteria or yeast) capable of synthesizing the desired molecules (e.g. biopolymers e.g. polypeptides), may be generated and placed within the particles. The pore size of the outer membrane can be selected such that the molecule of interest (e.g. recombinant polypeptide) exits the particle into the surrounding medium, but the genetically engineered bacteria cannot. This allows for easy extraction and purification of the molecules thereby increasing efficiency and overall yield. Furthermore, since the particles may be used in production batches working in continuous process modes, the time interval between the production batches may be reduced.

Thus, according to one aspect of the present invention there is provided a particle comprising: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth, (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms, and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water.

The term "particle" as used herein refers to an enclosed structure (e.g. capsule). The particle of the present invention may be of various shapes and sizes depending on the intended use of the particle (described in further detail below). Thus, the particle may be about 0.5 cm, 1 cm, 2 cm, 3 cm, 4 cm, 5 cm, 6 cm, 7 cm, 8 cm, 9 cm, 10 cm, 11 cm, 12 cm, 13 cm, 14 cm, 15 cm, 16 cm, 17 cm, 18 cm, 19 cm, 20 cm, 25 cm, 30 cm, 35 cm or 40 cm in length.

The term "microorganism" as used herein refers to an organism which is only visible using a microscope. The organism of the present invention can be a eukaryotic

organism (e.g., protozoa, algae or fungi for example yeast) or a prokaryotic organism (e.g., bacteria or archaea). The microorganisms of the present invention may be in any cellular environment, such as for example, in a biofilm, as isolated cells or as a cell suspension.

Exemplary bacteria which may be comprised in the particle of the present invention include gram positive bacteria and gram negative bacteria (see also list in Tables 1A-D, below).

The term "Gram-positive bacteria" as used herein refers to bacteria characterized by having as part of their cell wall structure peptidoglycan as well as polysaccharides and/or teichoic acids and are characterized by their blue-violet color reaction in the Gram-staining procedure. Representative Gram-positive bacteria include: *Actinomyces* spp., *Bacillus anthracis*, *Bifidobacterium* spp., *Clostridium botulinum*, *Clostridium perfringens*, *Clostridium* spp., *Clostridium tetani*, *Corynebacterium diphtheriae*, *Corynebacterium jeikeium*, *Enterococcus faecalis*, *Enterococcus faecium*, *Erysipelothrix rhusiopathiae*, *Eubacterium* spp., *Gardnerella vaginalis*, *Gemella morbillorum*, *Leuconostoc* spp., *Mycobacterium abscessus*, *Mycobacterium avium* complex, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium haemophilum*, *Mycobacterium kansasii*, *Mycobacterium leprae*, *Mycobacterium marinum*, *Mycobacterium scrofulaceum*, *Mycobacterium smegmatis*, *Mycobacterium terrae*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Nocardia* spp., *Peptococcus niger*, *Peptostreptococcus* spp., *Propionibacterium* spp., *Sarcina lutea*, *Staphylococcus aureus*, *Staphylococcus auricularis*, *Staphylococcus capitis*, *Staphylococcus cohnii*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus lugdanensis*, *Staphylococcus saccharolyticus*, *Staphylococcus saprophyticus*, *Staphylococcus schleiferi*, *Staphylococcus similans*, *Staphylococcus warneri*, *Staphylococcus xylosus*, *Streptococcus agalactiae* (group B streptococcus), *Streptococcus anginosus*, *Streptococcus bovis*, *Streptococcus canis*, *Streptococcus equi*, *Streptococcus milleri*, *Streptococcus mitior*, *Streptococcus mutans*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* (group A streptococcus), *Streptococcus salivarius*, *Streptococcus sanguis*.

The term "Gram-negative bacteria" as used herein refer to bacteria characterized by the presence of a double membrane surrounding each bacterial cell. Representative

Gram-negative bacteria include *Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, *Actinobacillus actinomycetemcomitans*, *Aeromonas hydrophila*, *Alcaligenes xylosoxidans*, *Bacteroides*, *Bacteroides fragilis*, *Bartonella bacilliformis*, *Bordetella* spp., *Borrelia burgdorferi*, *Branhamella catarrhalis*, *Brucella* spp., *Campylobacter* spp., *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Chromobacterium violaceum*, *Citrobacter* spp., *Eikenella corrodens*, *Enterobacter aerogenes*, *Escherichia coli*, *Flavobacterium meningosepticum*, *Fusobacterium* spp., *Haemophilus influenzae*, *Haemophilus* spp., *Helicobacter pylori*, *Klebsiella pneumoniae*, *Klebsiella* spp., *Legionella* spp., *Leptospira* spp., *Moraxella catarrhalis*, *Morganella morganii*, *Mycoplasma pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pasteurella multocida*, *Plesiomonas shigelloides*, *Prevotella* spp., *Proteus* spp., *Providencia rettgeri*, *Pseudomonas aeruginosa*, *Pseudomonas* spp., *Rickettsia prowazekii*, *Rickettsia rickettsii*, *Rochalimaea* spp., *Salmonella* spp., *Salmonella typhi*, *Serratia marcescens*, *Shigella* spp., *Shigella sonnei*, *Treponema carateum*, *Treponema pallidum*, *Treponema pallidum endemicum*, *Treponema pertenue*, *Veillonella* spp., *Vibrio cholerae*, *Vibrio vulnificus*, *Yersinia enterocolitica*, *Yersinia pestis*.

The term "fungi" as used herein refers to the heterotrophic organisms characterized by the presence of a chitinous cell wall, and in the majority of species, filamentous growth as multicellular hyphae. Representative fungi which may be comprised in the particle of the present invention include *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *Candida dubliniensis* (see also list in Tables 1A-D, below).

The term "yeast" as used herein refers to the eukaryotic micro-organisms classified in the kingdom Fungi. Representative yeast which may be comprised in the particle of the present invention include *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, *Rhodotorula rubra*, *Torulopsis* and *Trichosporon cutaneum* (see also list in Tables 1A-D, below).

The term "algae" as used herein refers to the simple, typically autotrophic eukaryotic organisms. Representative algae which may be comprised in the particle of the present invention include *Chlorella*, *Chlamdomonas*, *Chaetoceros*, *Spirolina*, *Dunaliella* and *Porphyrillum*.

It will be appreciated that selection of the microorganisms used will be determined according to the intended use of the particle (described in further detail below). For example, if the particle is used for petroleum wastewater treatment the microorganisms used may be selected from the list detailed in Table 1A, below. Alternatively, if the particle is used for municipal wastewater treatment, for food industry wastewater treatment or for pharmaceutical wastewater treatment the microorganisms used may be selected from the non-limiting lists detailed in Tables 1B-D, respectively, below or a combination thereof.

| Table 1A: List of microorganisms for use in petroleum wastewater treatment |                                                                                                                                                                              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
|----------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Yeast                                                                      | Fungi                                                                                                                                                                        | Bacterial                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| 1. <i>Sporobolomyces</i> .<br>2. <i>Trichosporon</i> .                     | 1. <i>Penicillium</i> .<br>2. <i>Cunninghamella</i> .<br>3. <i>Verticillium</i> .<br>4. <i>Rhodospiridium</i> .<br>6. <i>Brevibacterium</i> .<br>7. <i>Corynebacterium</i> . | 1. <i>Bacillus megaterium</i> (ATCC 14581).<br>2. <i>Bacillus brevis</i> (ATCC 8246).<br>3. <i>Bacillus subtilis</i> .<br>4. <i>Bacillus pumilis</i> .<br>5. <i>Bacillus firmus</i> .<br>6. <i>Bacillus licheniformis</i> .<br>7. <i>Escherichia coli</i> (ATCC 33456).<br>8. <i>Enterobacter aerogenes</i> .<br>9. <i>Pseudomonas putida</i> spp. (ATCC 12633).<br>10. <i>Pseudomonas stutzeri</i> AN10.<br>11. <i>Pseudomonas alcaligenes</i> .<br>12. <i>Micrococcus luteus</i> (ATCC 4698).<br>13. <i>Micrococcus lylae</i><br>14. <i>Stenotrophomonas maltophilia</i> (ATCC 12714, 13270, 14535, 17666).<br>15. <i>Acinetobacter baumannii</i> .<br>16. <i>Acinetobacter baumannii</i> (ATCC19606).<br>17. <i>Arthrobacter</i> spp.<br>18. <i>Achromobacter</i> spp.<br>19. <i>Nocardia asteroides</i> (ATCC10904).<br>20. <i>Nocardia</i> spp. (ATCC12288).<br>21. <i>Rhodotrulla</i> spp.<br>22. <i>Beauveria bassiana</i> .<br>23. <i>Burkholderia cepacia</i> .<br>24. <i>Mortierella</i> spp.<br>25. <i>Flavobacterium</i> spp. |

| Table 1B: List of microorganisms for use in municipal wastewater treatment |                                                           |                                         |
|----------------------------------------------------------------------------|-----------------------------------------------------------|-----------------------------------------|
| Specific species                                                           | Material biodegradation and/or biosorption and/or habitat | Type of microorganisms                  |
|                                                                            | phosphorus removal                                        | <i>Candidatus Accumulibacter</i>        |
|                                                                            |                                                           | <i>Candidatus Microthrix parvicella</i> |
|                                                                            |                                                           | <i>Candidatus Nostocoida limicola</i>   |
|                                                                            |                                                           | <i>Rhodocyclus</i> species              |
| <i>Micrococcus phosphovorans</i>                                           |                                                           | Actinobacteria species                  |
| <i>Tetrasphaera elongata</i>                                               |                                                           |                                         |
| <i>Tetrasphaera australiensis</i>                                          |                                                           |                                         |
| <i>Tetrasphaera japonica</i>                                               |                                                           |                                         |
| <i>Tessaracoccus bendigoensis</i>                                          |                                                           |                                         |
| <i>Nitrosomonas europaea</i>                                               | Nitrification                                             | <i>Nitrosomonas</i>                     |
|                                                                            |                                                           | <i>Nitrobacter</i>                      |
| <i>Bacillus subtilis</i>                                                   |                                                           | Bacillus Species                        |
| <i>Bacillus megaterium</i>                                                 |                                                           |                                         |
| <i>Bacillus thuringiensis</i>                                              |                                                           |                                         |
| <i>Bacillus stearothermophilus</i>                                         |                                                           |                                         |
| <i>Bacillus licheniformis</i>                                              |                                                           |                                         |
| <i>Bacillus polymyxa</i>                                                   |                                                           |                                         |
| <i>Bacillus sp.</i>                                                        |                                                           |                                         |
| <i>Bacillus pumilus</i>                                                    |                                                           |                                         |
| <i>Bacillus coagulans (S1)</i>                                             |                                                           |                                         |
|                                                                            |                                                           | <i>Serratia marcescens</i>              |
| <i>Pseudomonas fluorescens</i>                                             | Oil, fats & Grease (FOG)                                  | <i>Pseudonocardia</i> Species           |
| <i>Pseudomonas stutzeri</i>                                                |                                                           |                                         |
|                                                                            |                                                           | <i>Burkholderia</i> Species             |
|                                                                            |                                                           | <i>Acinetobacter</i> Species            |
|                                                                            |                                                           | <i>Escherichia</i> Species              |
| <i>Arthrobacter sp. N3</i>                                                 |                                                           | <i>Arthrobacter</i>                     |
|                                                                            |                                                           | <i>Enterobacter aerogenes</i> E13       |
|                                                                            |                                                           | <i>Lactobacillus sporogenes</i>         |
|                                                                            |                                                           | <i>Cellulomonas uda</i>                 |
|                                                                            |                                                           | <i>Micrococcus sp.</i>                  |
|                                                                            | Phenols                                                   | <i>Thiobacillus novellus</i>            |
|                                                                            |                                                           | <i>Burkholderia</i> sp.                 |
|                                                                            |                                                           | <i>Pseudomonas putida</i> Species       |
|                                                                            |                                                           | <i>Sphingomonas xenophaga</i>           |
| <i>Yarrowia</i>                                                            | Oil, fats & Grease (FOG)                                  | Fungi                                   |
| <i>Rhodotorula</i>                                                         |                                                           |                                         |
|                                                                            |                                                           |                                         |
| <i>Saccharomyces cerevisiae</i>                                            | Oil, fats & Grease (FOG)                                  | Yeast                                   |
| <i>Candida</i>                                                             |                                                           |                                         |
| <i>Candida tropicalis</i>                                                  | Phenols                                                   |                                         |
|                                                                            |                                                           |                                         |

| Table 1C: List of microorganisms for use in food industry wastewater treatment                                                                                                                                                                                                                                                                                                     |                                                           |                                                 |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------|-------------------------------------------------|
| Specific species                                                                                                                                                                                                                                                                                                                                                                   | Material biodegradation and/or biosorption and/or habitat | Type of microorganisms                          |
| <i>S. roseus</i><br><i>S. hispanicus</i>                                                                                                                                                                                                                                                                                                                                           | Halophilic bacteria                                       | <i>Salinicoccus</i>                             |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Nesterenkonia halobia</i>                    |
| <i>A. mortivallis</i><br><i>A. iraqi</i>                                                                                                                                                                                                                                                                                                                                           |                                                           | <i>Actinopolyspora halophila</i>                |
| <i>N. lucentensis</i><br><i>N. halophila</i>                                                                                                                                                                                                                                                                                                                                       |                                                           | <i>Nocardiopsis</i>                             |
| <i>Halomonas halodenitrificans</i><br><i>H. elongata</i><br><i>H. subglaciescola</i><br><i>H. halodurans</i><br><i>H. halmophila</i><br><i>H. eurihalina</i><br><i>H. halophila</i><br><i>H. salina</i><br><i>H. halodenitrificans</i><br><i>H. variabilis</i><br><i>H. canadensis</i><br><i>H. israelensis</i><br><i>H. pantelleriense</i><br><i>Chromohalobacter marismortui</i> |                                                           | <i>Halobacteriaceae</i>                         |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Salinivibrio costicola</i>                   |
| <i>B. halophilus</i><br><i>B. salexigens</i>                                                                                                                                                                                                                                                                                                                                       |                                                           | <i>Bacillus</i> species                         |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Pseudomonas beijerinckii</i>                 |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Pseudomonas halophila</i>                    |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Tetragenococcus</i>                          |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Marinococcus halophilus</i>                  |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Vibrio (Salinivibrio) costicola</i>          |
| <i>Micrococcus halobius</i><br><i>Micrococcus albus</i>                                                                                                                                                                                                                                                                                                                            |                                                           | <i>Micrococcus</i>                              |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Paracoccus (Halomonas) halodenitrificans</i> |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Flavobacterium (Halomonas) halmephilum</i>   |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Flavobacterium gondwanense</i>               |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Flavobacterium salegens</i>                  |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Planococcus (Marinococcus) halophilus</i>    |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Spirochaeta halophila</i>                    |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Arhodomonas aquaeolei</i>                    |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Dichotomicrobium thermohalophilum</i>        |
| <i>S. roseus</i><br><i>S. hispanicus</i>                                                                                                                                                                                                                                                                                                                                           |                                                           | <i>Salinicoccus</i>                             |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Nesterenkonia halobia</i>                    |
| <i>A. mortivallis</i><br><i>A. iraqi</i>                                                                                                                                                                                                                                                                                                                                           |                                                           | <i>Actinopolyspora halophila</i>                |
| <i>N. lucentensis</i><br><i>N. halophila</i>                                                                                                                                                                                                                                                                                                                                       |                                                           | <i>Nocardiopsis</i>                             |
| <i>Nitrosomonas europaea</i>                                                                                                                                                                                                                                                                                                                                                       | Nitrification                                             | <i>Nitrosomonas</i>                             |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                           | <i>Nitrobacter</i>                              |
|                                                                                                                                                                                                                                                                                                                                                                                    | Oil, fats & Grease (FOG)                                  |                                                 |
| <i>Bacillus subtilis</i>                                                                                                                                                                                                                                                                                                                                                           |                                                           | <i>Bacillus</i> Species                         |
| <i>Bacillus megaterium</i>                                                                                                                                                                                                                                                                                                                                                         |                                                           |                                                 |
| <i>Bacillus thuringiensis</i>                                                                                                                                                                                                                                                                                                                                                      |                                                           |                                                 |

|                                    |                |                                   |
|------------------------------------|----------------|-----------------------------------|
| <i>Bacillus stearothermophilus</i> |                |                                   |
| <i>Bacillus licheniformis</i>      |                |                                   |
| <i>Bacillus polymyxa</i>           |                |                                   |
| <i>Bacillus sp.</i>                |                |                                   |
| <i>Bacillus pumilus</i>            |                |                                   |
| <i>Bacillus coagulans (S1)</i>     |                |                                   |
|                                    |                |                                   |
| <i>Pseudomonas fluorescens</i>     |                | <i>Serratia marcescens</i>        |
| <i>Pseudomonas stutzeri</i>        |                | <i>Pseudonocardia</i> Species     |
|                                    |                |                                   |
|                                    |                | <i>Burkholderia</i> Species       |
|                                    |                | <i>Acinetobacter</i> Species      |
|                                    |                | <i>Escherichia</i> Species        |
| <i>Arthrobacter sp. N3</i>         |                | <i>Arthrobacter</i>               |
|                                    | Organic matter | <i>Enterobacter aerogenes E13</i> |
|                                    |                | <i>Lactobacillus sporogenes</i>   |
|                                    |                | <i>Cellulomonas uda</i>           |
|                                    |                | <i>Micrococcus sp.</i>            |
|                                    |                | <i>Thiobacillus novellus</i>      |
|                                    |                | <i>Bifidobacteria</i>             |
| <i>B. longum</i>                   |                |                                   |
| <i>B. thermophilum</i>             |                |                                   |
| <i>B. animalis</i>                 |                |                                   |
| <i>B. pseudolongum</i>             |                |                                   |
| <i>B. infantis</i>                 |                |                                   |
| <i>B. adolescentis</i>             |                |                                   |
| <i>B. angulatum</i>                |                |                                   |
| <i>B. animalis</i>                 |                |                                   |
| <i>B. asteroides</i>               |                |                                   |
| <i>B. bifidum</i>                  |                |                                   |
| <i>B. boum</i>                     |                |                                   |
| <i>B. breve</i>                    |                |                                   |
| <i>B. catenulatum</i>              |                |                                   |
| <i>B. choerinum</i>                |                |                                   |
| <i>B. coryneforme</i>              |                |                                   |
| <i>B. cuniculi</i>                 |                |                                   |
| <i>B. dentium</i>                  |                |                                   |
| <i>B. gallicum</i>                 |                |                                   |
| <i>B. gallinarum</i>               |                |                                   |
| <i>B. globosum</i>                 |                |                                   |
| <i>B. indicum</i>                  |                |                                   |
| <i>B. lactis</i>                   |                |                                   |
| <i>B. magnum</i>                   |                |                                   |
| <i>B. merycicum</i>                |                |                                   |
| <i>B. minimum</i>                  |                |                                   |
| <i>B. pseudocatenulatum</i>        |                |                                   |
| <i>B. pseudolongum</i>             |                |                                   |
| <i>B. pullorum</i>                 |                |                                   |
| <i>B. ruminatum</i>                |                |                                   |
| <i>B. saeculare</i>                |                |                                   |
| <i>B. subtile</i>                  |                |                                   |
| <i>B. suis</i>                     |                |                                   |
| <i>B. thermophilum</i>             |                |                                   |
| <i>Enterococcus faecalis FO1</i>   | Organic matter | <i>Enterococcus</i>               |
| <i>Enterococcus faecium FJ2</i>    |                |                                   |
| <i>Enterococcus FO5</i>            |                |                                   |
| <i>Enterococcus RE9</i>            |                |                                   |
| <i>Enterococcus sp.</i>            |                |                                   |
| <i>Arthrobacter globiformis</i>    |                | <i>Arthrobacter</i>               |
| <i>Aureobacterium liquefaciens</i> |                | <i>Aureobacterium</i>             |
| <i>Brevibacterium casei</i>        |                | <i>Brevibacterium</i>             |
| <i>Brevibacterium linens</i>       |                |                                   |
| <i>Cellulomonas uda</i>            |                | <i>Cellulomonas</i>               |

|                                                                                                                                                                                                                                                                                 |                          |                                      |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|--------------------------------------|
| <i>Microbacterium arborescens</i><br><i>Microbacterium lacticum</i>                                                                                                                                                                                                             |                          | <i>Microbacterium</i>                |
| <i>Micrococcus lylae</i><br><i>Micrococcus freudenreichii</i><br><i>Micrococcus kristinae</i><br><i>Micrococcus varians</i>                                                                                                                                                     |                          | <i>Micrococcus</i>                   |
|                                                                                                                                                                                                                                                                                 |                          | <i>P. freudenreichii</i>             |
| <i>Propionibacterium jensenii</i><br><i>Propionibacterium acidipropionici</i><br><i>Propionibacterium sp.</i><br><i>Propionibacterium thoenii</i>                                                                                                                               |                          | <i>Propionibacterium</i>             |
|                                                                                                                                                                                                                                                                                 |                          | <i>Rhodococcus chlorophenolicus</i>  |
|                                                                                                                                                                                                                                                                                 |                          | <i>Streptococcus thermophilus</i> S3 |
|                                                                                                                                                                                                                                                                                 |                          | <i>Pseudomonas species</i>           |
| <i>Lactobacillus acidophilus</i><br><i>Lactobacillus casei</i><br><i>Lactobacillus GD1</i><br><i>Lactobacillus lactis</i><br><i>Lactobacillus plantarum</i><br><i>Lactobacillus rhamnosus</i><br><i>Lactococcus Bu2-60B</i><br><i>Lactobacillus delbrueckii ssp. bulgaricus</i> |                          | <i>Lactobacillus</i>                 |
| <i>Yarrowia</i>                                                                                                                                                                                                                                                                 | Oil, fats & Grease (FOG) | Fungi                                |
| <i>Rhodotorula</i>                                                                                                                                                                                                                                                              |                          |                                      |
|                                                                                                                                                                                                                                                                                 |                          |                                      |
| <i>Saccharomyces cerevisiae</i><br><i>Candida</i>                                                                                                                                                                                                                               | Oil, fats & Grease (FOG) | Yeast                                |

Table 1D: List of microorganisms for use in pharmaceutical wastewater treatment

| Specific species                                                                                                                                                         | Material biodegradation and/or biosorption and/or habitat                                | Type of microorganisms                              |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|-----------------------------------------------------|
|                                                                                                                                                                          | phosphorus removal                                                                       | <i>Candidatus Accumulibacter</i>                    |
|                                                                                                                                                                          |                                                                                          | <i>Candidatus Microthrix parvicella</i>             |
|                                                                                                                                                                          |                                                                                          | <i>Candidatus Nostocoida limicola</i>               |
|                                                                                                                                                                          |                                                                                          | <i>Rhodocyclus species</i>                          |
| <i>Microthrix phosphovorus</i><br><i>Tetrasphaera elongata</i><br><i>Tetrasphaera australiensis</i><br><i>Tetrasphaera japonica</i><br><i>Tessaracoccus bendigoensis</i> |                                                                                          | <i>Actinobacteria species</i>                       |
|                                                                                                                                                                          |                                                                                          |                                                     |
|                                                                                                                                                                          | polycyclic aromatic hydrocarbons - benzene, phenol, and toluene.                         | <i>Burkholderia sp.</i>                             |
|                                                                                                                                                                          | polycyclic aromatic hydrocarbons - benzene, phenol, and toluene. Trichloroethylene (TCE) | <i>Pseudomonas putida</i> Species                   |
|                                                                                                                                                                          | phenol                                                                                   | <i>Sphingomonas xenophaga</i>                       |
|                                                                                                                                                                          |                                                                                          | <i>Sphingomonas cloacae</i>                         |
|                                                                                                                                                                          |                                                                                          | <i>Bacillus weihenstephanensis</i> strain RBE1CD    |
|                                                                                                                                                                          |                                                                                          | <i>B. sphaericus</i> strain D45                     |
|                                                                                                                                                                          |                                                                                          | <i>B. cereus</i>                                    |
|                                                                                                                                                                          |                                                                                          | <i>Enterobacter cancerogenus</i> sp. EBD            |
|                                                                                                                                                                          |                                                                                          | <i>Acinetobacter sp.</i>                            |
|                                                                                                                                                                          |                                                                                          | <i>Acinetobacter calcoaceticus</i> strain CBMAI 464 |
|                                                                                                                                                                          |                                                                                          | <i>Pseudomonas sp. Hugh2319</i>                     |

|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       |                                                                 |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------|-----------------------------------------------------------------|
|                                                                                                                                                                                                                                                                                                                                                                                    | pyridine                                              | <i>Bacillus weihenstephanensis</i> strain RBE1CD                |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Bacillus sphaericus</i> strain D45                           |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Bacillus cereus</i>                                          |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Acinetobacter</i> sp.                                        |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Acinetobacter calcoaceticus</i> strain CBMAI 464             |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Pseudomonas</i> sp. Hugh2319                                 |
| <i>S. roseus</i><br><i>S. hispanicus</i>                                                                                                                                                                                                                                                                                                                                           | Halophilic bacteria                                   | <i>Salinicoccus</i>                                             |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Nesterenkonia halobia</i>                                    |
| <i>A. mortivallis</i><br><i>A. iraqi</i>                                                                                                                                                                                                                                                                                                                                           |                                                       | <i>Actinopolyspora halophila</i>                                |
| <i>Halomonas halodenitrificans</i><br><i>H. elongata</i><br><i>H. subglaciescola</i><br><i>H. halodurans</i><br><i>H. halmophila</i><br><i>H. eurihalina</i><br><i>H. halophila</i><br><i>H. salina</i><br><i>H. halodenitrificans</i><br><i>H. variabilis</i><br><i>H. canadensis</i><br><i>H. israelensis</i><br><i>H. pantelleriense</i><br><i>Chromohalobacter marismortui</i> |                                                       | <i>Halobacteriaceae</i>                                         |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Salinivibrio costicola</i>                                   |
| <i>B. halophilus</i><br><i>B. salexigens</i>                                                                                                                                                                                                                                                                                                                                       |                                                       | <i>Bacillus</i> species                                         |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Pseudomonas beijerinckii</i>                                 |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Pseudomonas halophila</i>                                    |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Tetragenococcus</i>                                          |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Marinococcus halophilus</i>                                  |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Vibrio</i> ( <i>Salinivibrio</i> ) <i>costicola</i>          |
| <i>Micrococcus halobius</i><br><i>Micrococcus albus</i>                                                                                                                                                                                                                                                                                                                            |                                                       | <i>Micrococcus</i>                                              |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Paracoccus</i> ( <i>Halomonas</i> ) <i>halodenitrificans</i> |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Flavobacterium</i> ( <i>Halomonas</i> ) <i>halmophilum</i>   |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Flavobacterium gondwanense</i>                               |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Flavobacterium salegens</i>                                  |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Planococcus</i> ( <i>Marinococcus</i> ) <i>halophilus</i>    |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Spirochaeta halophila</i>                                    |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Arhodomonas aquaeolei</i>                                    |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Dichotomicrobium thermohalophilum</i>                        |
| <i>N. lucentensis</i><br><i>N. halophila</i>                                                                                                                                                                                                                                                                                                                                       |                                                       | <i>Nocardiopsis</i>                                             |
|                                                                                                                                                                                                                                                                                                                                                                                    | Toluene                                               | <i>Burkholderia cepacia</i>                                     |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Ralstonia pickettii</i>                                      |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Pseudomonas mendocina</i>                                    |
| <i>Thauera aromatic</i> K172<br><i>T. aromatica</i> T1                                                                                                                                                                                                                                                                                                                             |                                                       | <i>Thauera</i> sp. Strain DNT-1                                 |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Azoarcus</i> sp. strain T                                    |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Rhodopseudomonas palustris</i>                               |
|                                                                                                                                                                                                                                                                                                                                                                                    |                                                       | <i>Azoarcus evansii</i>                                         |
|                                                                                                                                                                                                                                                                                                                                                                                    | Nitrogen (denitrification), ethanol (2-chloroethanol) | <i>Pseudomonas stutzeri</i>                                     |
|                                                                                                                                                                                                                                                                                                                                                                                    | Ether                                                 | <i>Pseudonocardia</i> sp. Strain ENV478                         |
|                                                                                                                                                                                                                                                                                                                                                                                    | Organic solvents - vinyl chloride                     | <i>Arthrobacter</i> Species                                     |

|                                                   |                                                                                                                           |                                      |
|---------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------|--------------------------------------|
|                                                   | (VC), tetrachloroethene (PCE) and trichloroethene (TCE), ethene (ETH)                                                     |                                      |
|                                                   |                                                                                                                           | <i>Pseudonocardia</i> Species        |
|                                                   |                                                                                                                           | <i>Mycobacterium</i> Species         |
|                                                   | Acetylene                                                                                                                 | Methanogens                          |
|                                                   | Methylene chloride, chloroform, trichloroethylene, ethylene.                                                              | <i>Methanotrophic consortium</i>     |
|                                                   | Organic solvents - <i>tert</i> -amyl methyl ether and <i>tert</i> -amyl alcohol                                           | <i>Mycobacterium austroafricanum</i> |
| <i>Nitrosomonas europaea</i>                      | Nitrification                                                                                                             | <i>Nitrosomonas</i>                  |
|                                                   |                                                                                                                           | <i>Nitrobacter</i>                   |
|                                                   | BTEX                                                                                                                      | <i>Bacillus hexavarbovorum</i>       |
|                                                   |                                                                                                                           | <i>Pseudomonas</i> Species           |
|                                                   |                                                                                                                           | <i>Burkholderia</i> Species          |
|                                                   |                                                                                                                           | <i>Arthrobacter</i> Species          |
|                                                   |                                                                                                                           | <i>Alcaligen</i> Species             |
|                                                   |                                                                                                                           | <i>Corynebacterium</i> Species       |
|                                                   |                                                                                                                           | <i>Flavobacterium</i> Species        |
|                                                   |                                                                                                                           | <i>Norcadia</i> Species              |
|                                                   |                                                                                                                           | <i>Achromobacter</i> Species         |
|                                                   |                                                                                                                           | <i>Micrococcus</i> Species           |
|                                                   | Sulfate, methanol                                                                                                         | <i>Desulfotomaculum kuznetsovii</i>  |
|                                                   | Organic solvents - Acetone, cyclohexane, styrene, benzene, ethylbenzene, propylbenzene, dioxane, and 1,2-dichloroethylene | <i>Mycobacterium vaccae</i>          |
|                                                   | Organic solvents                                                                                                          | <i>Bacillus</i> Species              |
|                                                   |                                                                                                                           | <i>Nocardia</i> Species              |
|                                                   | Organic solvents - short-chain halogenated aliphatic hydrocarbons.                                                        | <i>Xanthobacter autotrophicus</i>    |
|                                                   | Organic solvents - Ethanol                                                                                                | <i>Desulfosporosinus</i> Species     |
|                                                   | Organic solvents - Isopropanol (IPA)                                                                                      | <i>Bacillus pallidus</i>             |
|                                                   | Antibiotics                                                                                                               | <i>Escherichia coli</i>              |
|                                                   |                                                                                                                           | <i>Acinetobacter</i> spp.            |
|                                                   |                                                                                                                           | <i>Campylobacter</i> spp             |
|                                                   |                                                                                                                           | <i>Pseudomonas</i> spp               |
|                                                   |                                                                                                                           | <i>Enterococci</i>                   |
|                                                   |                                                                                                                           | <i>Mycobacterium</i> spp             |
|                                                   | decolorization of dye wastewaters                                                                                         | Fungi                                |
| white rot fungus<br>Phanerochaete<br>chrysosponum | Polycyclic aromatic hydrocarbons (PAH)                                                                                    |                                      |
| <i>Saccharomyces cerevisiae</i>                   | volatile organic compound (ethanol, ethyl acetate)                                                                        | Yeast                                |
| <i>Candida guilliermondii</i>                     | volatile organic compound (ethanol, ethyl acetate)                                                                        |                                      |
| <i>Candida tropicalis</i>                         | Phenol                                                                                                                    |                                      |
| <i>Saccharomyces cerevisiae</i>                   | Phthalic acid                                                                                                             |                                      |

Table 1E: List of microorganisms for use in De-nitrification treatment

| Specific species                | Type of microorganisms  |
|---------------------------------|-------------------------|
| <i>Pseudomonas stutzeri</i>     | <i>Alcaligenes</i>      |
|                                 | <i>Pseudomonas</i>      |
|                                 | <i>Methylobacterium</i> |
|                                 | <i>Bacillus</i>         |
| <i>Paracoccus denitrificans</i> | <i>Paracoccus</i>       |
|                                 | <i>Hyphomicrobium</i>   |
|                                 |                         |

According to one embodiment, the microorganisms comprised in the particle are a homogenous population.

According to another embodiment, the microorganisms comprised in the particle are a heterogeneous population.

Typically, the microorganisms of the present invention are dried (e.g. in a powder form) prior to encapsulation thereof.

Any suitable drying technology such as freeze-drying, spray drying, refractive windows drying (described for example. in U.S. Application No. 20070122397) drying under reduced pressure (described for example. in PCT Publication No. WO/2001/036590) may be used so long as the microorganisms are capable of propagating following activation (i.e. remain viable).

For example, freeze-drying may be carried out as described in detail in Example 1 of the Examples section which follows. In short, microorganisms (e.g. bacteria) are grown for a suitable length of time (e.g. overnight) in growth medium. The bacteria is collected (e.g. by centrifugation) and suspended (e.g. in PBS solution). The suspended culture is collected (e.g. by centrifuged) and suspended (e.g. in ice cold PBS comprising 5 % sucrose). The culture is incubated (e.g. at room temperature, 22 °C) for a short period of time (e.g. 20 minutes) and then incubated for several days (e.g. 48-72 h) at a freezing temperature (e.g. -80 °C). Finally, the culture is freeze-dried for a few days (e.g. 52 h) using a protective freeze agent (e.g. sucrose) and stored at room temperature (e.g. inside a dissector).

The present invention contemplates introduction of at least about  $10^6$ ,  $10^7$ ,  $10^8$ ,  $10^9$ ,  $10^{10}$ ,  $10^{11}$  or  $10^{12}$  microorganisms/ml into the particle. It will be appreciated that during the life span of the particle, the microorganism levels may increase or decrease (especially after activation of the particle as described below).

In order to support microorganism growth, the particle of this aspect of the present invention comprises a solid matrix of nutrients (also referred to herein, as the inner core).

As used herein the term "solid matrix" refers to any solid material which comprises a microorganism (e.g. bacterial) growth supportive capacity. Typically the inner core contains sufficient nutrients to facilitate viability and growth of the microorganisms contained within the particle for at least 5 days, 10 days, 20 days, 30 days, 40 days, 50 days, 60 days, 70 days, 80 days, 90 days, 100 days, 110 days, 120 days, 130 days, 140 days or more.

Particular compositions (e.g. matrixes and nutrients) suitable for use in growing microorganisms are well known in the art (see for example, Shijun Liu and Laurie Usinger,

[http://www\(dot\)sciencebuddies\(dot\)org/mentoring/project\\_ideas/MicroBio\\_Agar\(dot\)shtml](http://www(dot)sciencebuddies(dot)org/mentoring/project_ideas/MicroBio_Agar(dot)shtml), fully incorporated herein by reference). For example, the inner core may comprise an agar including e.g. Luria Agar (LA), LB (Luria Bertani) Agar, MacConkey Agar, Miller's LB Agar, Blood agar, Chocolate agar, Hektoen enteric agar (HE), mannitol salt agar (MSA) and the like, or gelatin.

According to the present teachings, the solid matrix may comprise additional nutrients which support microorganism growth and/or which augment the microorganism activity (e.g. decontaminating activity). Thus, the inner core may comprise, for example, a source of amino acids and nitrogen (e.g., beef, yeast extract, tryptone), a sugar or carbon source (e.g. glucose), water, various salts (e.g. NaCl), essential elements (e.g. iron, magnesium, nitrogen, phosphorus, and sulfur), other compounds (e.g. lactate), peptides, proteins, carbohydrates and enzymes (e.g. degradation enzymes). The inner core may additionally comprise any nutrient needed for the microorganism growth and prosperity.

According to one embodiment, the inner core is dried prior to encapsulation thereof so as to prevent unwanted activation of the microorganisms. The inner core may be dried according to any suitable drying method known to one of ordinary skill in the art. Thus, for example, the inner core may be air-dried, dried in biological hood or vacuum-dried.

The inner core may be coated with a control release polymer such as a sustained release polymer which may control the rate of release of the nutrients from the inner core. Such polymers may include, without being limited to, a polyvinyl acetate (PVA)-based material, Kollidon SR (PVA/PVP matrix), Kollicoat SR 30D (30 % aqueous dispersion of polyvinyl acetate stabilized with polyvinyl pyrrolidone), chitozan, polylactic-co-glycolic acid (PLGA), PLGA with Polylactic acid (PLA) or combination of PLGA and PLA.

It will be appreciated that the particle of the present invention comprises an inner membrane which encapsulates the inner core and the dried microorganisms. The inner membrane of the present invention is typically fabricated from a water-soluble polymer.

As used herein, the phrase "water-soluble polymer" refers to a polymer that dissolves in an aqueous medium after at least one week of incubation therein, more preferably after 5 day incubation and even more preferably after 1 day incubation.

The water-soluble polymer may be a natural water-soluble polymer or a synthetic water-soluble polymer. Examples of such include, but are not limited to, gelatin, agar, polyethylene glycol, acrylic acid polymers, polysaccharide, polysaccharide gum and sodium alginate. It will be appreciated that the inner membrane may be fabricated from one polymer, from two polymers, from three polymers or from several polymers as can be determined by one of ordinary skill in the art.

The thickness of the inner membrane may be from about 50  $\mu\text{m}$  to about 800  $\mu\text{m}$ .

According to a specific embodiment, the inner membrane is fabricated from gelatin.

It will be appreciated that additional elements may be incorporated into the inner membrane. These may include glass or polymer beads, activated carbon granules, activated carbon chips, control release oxygen compounds; sustained release oxygen compounds, enzymes and nutrients (see list hereinabove).

According to one embodiment, the inner membrane further comprises an oxygen release compound (i.e. oxygen carrier) within. Such a compound can increase the dissolved oxygen within the particles. It will be appreciated that the oxygen release compound can be selected for sustained release such that it controls the rate of oxygen release from the inner core.

Alternatively, a particle may be fabricated with only an oxygen release compound within (i.e. only oxygen carrier without bacteria) in order to increase oxygen levels in an area of interest (e.g. in treated wastewater, as detailed below). In such cases, a particle is generated such that the inner core comprises a solid oxygen release compound, the inner core is surrounded by an inner membrane being fabricated from a water-soluble polymer, and an outer porous membrane being insoluble in water is fabricated to surround the inner membrane.

As used herein the phrase “oxygen carrier” refers to a molecule capable of transporting, delivering and/or supplying oxygen to the microorganisms or to an area of interest, thus providing aerobic conditions.

The oxygen carrier may be thus embedded within or covalently attached to the inner core. Covalent attachment of the oxygen carrier to the inner membrane may be via, for example a tethering molecule such as Poly [Ethylene Glycol]).

The oxygen carrier can be incorporated into the inner membrane by various ways. For example, the oxygen carrier may be mixed with the polymers fabricating the inner membrane (e.g., gelatin) and be subjected to the solidification process forming the inner membrane such that it is embedded within the inner membrane. For example, if the inner membrane is formed by a gel suspension, the oxygen carrier can be mixed with the gel's solution and be subjected to the solidification process casting the gel. Additionally or alternatively, if electro-spinning is employed in order to form the inner core, the oxygen carrier may be mixed with the polymeric solutions prior to the electro-spinning process. Still additionally or alternatively, the oxygen carrier may be covalently bound to the inner membrane using for example, a cross linking agent or an energy source.

An exemplary oxygen release compound which may be used in accordance with the present teachings includes, but is not limited to, ORC advanced® – oxygen release compound, available from REGENESIS, O-SOX™ (DGSI) available from Durham Geo-Enterprises Inc., PermeOx Plus® available from FMC corporation, OxyCal available from REX-BAC-T technologies and EOx™ available from EOS Remediation LLC.

The material of the inner membrane and components comprised therein may be selected to support biofilm formation.

As used herein the term "biofilm" refers to is an aggregate of microorganisms in which cells adhere to each other and/or to a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) in which microorganisms are dispersed and/or form colonies. The biofilm typically is made of polysaccharides and other macromolecules.

Biofilm formation may occur, for example, on the surface of the inner core (e.g. on the solid matrix), on the inside surface of the inner membrane (e.g. on the inner surface of the gelatin particle), as well as on the additional components (e.g. on the surface of the glass beads or on the surface of the carbon granules/chips).

According to one embodiment, the bacterial strain selected to induce biofilm formation is *Bacillus Subtilis*.

Thus the number of additional components may be selected according to the level of biofilm formation required.

In addition, the number of additional components may be selected according to the weight/buoyancy required.

As mentioned, the particle of the present invention further comprises an outer porous membrane surrounding the inner membrane.

The outer porous membrane is typically water-insoluble (i.e. the pore size of the membrane should not change following incubation in water). In addition, the porous membrane needs to withstand harsh unstable environments (e.g. pH variations, the presence of various solvents, etc.).

In order to prevent clogging of the pores, the outer porous membrane may be fabricated from a material that is resistant to biofilm formation.

There is wide variety of polymers that may be used to fabricate the porous outer membrane according to this aspect of the present invention. Below is a list of such polymers (see Table 2 for possible vendors of same): PVAL (polyvinyl-alcohol), Polyethersulfone (PES), Cellulose Acetate, Cellulose Nitrate, Ethyl Cellulose, Nitrocellulose Mixed Esters, Polycarbonate film, Nylon, PVDF(poly(vinylidene fluoride)), Polysulfone, PVOH, polyacrylamide, Poly(4-vinyl-N-alkylpyridinium bromide), poly(methacryloyloxydodecylpyridinium bromide, N-alkylated poly(4-vinyl pyridine), Poly(vinyl-N-hexylpyridinium), poly(N-alkyl vinylpyridine), poly(N-alkyl ethylene imine), poly(4-vinyl-N-alkylpyridinium bromide), poly(4-vinyl-N-

hexylpyridinium bromide), Poly (1-(chloromethyl).sub.4-vinylbenzene, Poly (Dimethyloctyl[4-vinylphenyl]methylammonium chloride), Poly (Dimethyldodecyl[4-vinylphenyl]methylammonium chloride), Poly (Dimethyltetradecyl[4-vinylphenyl]methylammonium chloride), 50:50 Poly (1-Chloromethyl)-4-vinylbenzene): Poly (Dimethyldodecyl[4-vinyl- phenyl]methylammonium chloride), 50:50 Poly (1-Chloromethyl)-4-vinylbenzene): Poly (Dimethyloctyl[4-vinylphenyl]methylammonium chloride), 50:50 Poly (Dimethyldodecyl[4-vinylphenyl]methylammonium chloride): Poly (Dimethyloctyl[4-vinylphenyl]methylammonium chloride), Poly (Tributyl-[4-vinylphenyl]methylphosphonium chloride) and Poly (Trioctyl-[4-vinylphenyl]methylphosphonium chloride).

**Table 2: Porous outer membrane polymers and vendors**

| Manufacture companies                                         | Raw materials                                             |
|---------------------------------------------------------------|-----------------------------------------------------------|
| Kuraray, Shanxi Sanwei Group                                  | PVAL (polyvinyl-alcohol)                                  |
| Sterlitech, Sigma-aldrich, General Electric Osmonics Labstore | Polyethersulfone (PES)                                    |
| Sterlitech, General Electric Osmonics Labstore                | Cellulose Acetate<br>Cellulose Nitrate<br>Ethyl cellulose |
| Sterlitech, General Electric Osmonics Labstore                | Nitrocellulose Mixed Esters                               |
| Sterlitech                                                    | Polycarbonate film                                        |
| Sterlitech                                                    | Nylon<br>PVDF(poly(vinylidene fluoride))                  |
|                                                               | Polysulfone                                               |

According to a specific embodiment the outer porous membrane is fabricated from Cellulose Acetate.

According to a specific embodiment the outer porous membrane is fabricated from Ethyl Cellulose.

The outer porous membrane of the particle may be fabricated uniformly of a single polymer, co-polymer or blend thereof. It is possible to form a porous membrane from a plurality of different polymers. There are no particular limitations to the number or arrangement of polymers used in forming the porous membrane. Any combination which is water-insoluble and enables formation of pores may be used. It is possible, for example, to apply polymers sequentially.

As mentioned, the pore size of the outer membrane is selected such that it allows trafficking of molecules of a particular size (e.g. water or proteins) while inhibiting trafficking of larger molecules (e.g. microorganisms). Thus, according to an embodiment of the present invention, the pore of the porous membrane is less than 0.95  $\mu\text{M}$ , 0.90  $\mu\text{M}$ , 0.85  $\mu\text{M}$ , less than 0.80  $\mu\text{M}$ , less than 0.75  $\mu\text{M}$ , less than 0.70  $\mu\text{M}$ , less than 0.65  $\mu\text{M}$ , less than 0.60  $\mu\text{M}$ , less than 0.55  $\mu\text{M}$ , less than 0.50  $\mu\text{M}$ , less than 0.45  $\mu\text{M}$ , less than 0.40  $\mu\text{M}$ , less than 0.35  $\mu\text{M}$ , less than 0.30  $\mu\text{M}$ , less than 0.25  $\mu\text{M}$ , or less than 0.20  $\mu\text{M}$ .

It will be appreciated that any method known in the art may be used to generate the particle of the present invention.

For example, according to the present teachings, the particle may be generated by inserting at least one inner core (i.e. solid matrix of nutrients) into the inner membrane. Any number of inner cores may be placed in the inner membrane as to support sustained microbial growth and proliferation. For example, 2 inner cores, 3 inner cores, 4 inner cores, 5 inner cores or more may be placed within the inner membrane. One of ordinary skill in the art will be able to determine the number of inner cores needed according to the intended use of the particle (described in further detail below).

It will be appreciated that for particular applications the particle does not have to comprise a solid matrix of nutrients. However, it is anticipated that according to this embodiment other elements are incorporated inside the inner membrane to enhance biofilm formation (e.g. the glass beads).

According to one embodiment, the inner membrane comprises dried nutrients in a powder form

According to another embodiment, the particle does not comprise nutrients, but relies on nutrients from the exterior culture medium. This may be particularly relevant for the application for synthesizing a molecule of interest, wherein the synthesis is effected in a reactor comprising a culture medium. In such circumstances, the nutrients required to support microorganism growth and prosperity may be supplied in the exterior culture medium (e.g. in the bioreactor).

Other components may also be added into the inner membrane such as the dried organisms (e.g. bacteria) and any additional components needed (e.g. glass beads, carbon granules/chips).

Once all the necessary components are added to the inner membrane, it is coated with a water-insoluble porous membrane (described in detail above). Coating the particle may be carried out by any method known in the art, as for example, by spraying, dripping, immersing etc. The thickness of the outer membrane may be from about 1  $\mu\text{m}$  to about 1000  $\mu\text{m}$ . Moreover, the particle may be sprayed several times on each side (e.g. 3-4 times) as needed to obtain the required thickness.

The particles for any of the below described applications may be pre-activated prior to use so as to transform the microorganisms comprised therein from a non-proliferating state to a proliferating state. Activating the population of microorganisms within the particle is effected by first contacting the particle with a liquid under conditions that allow the liquid to penetrate the outer porous membrane and wet the dried microorganisms

According to one embodiment, the particle is contacted with the liquid for a period of several hours to several days. According to a specific embodiment, the particle is contacted with the liquid for a period of about 24 to 96 hours.

The liquid substance which may be used to activate the microorganism may comprise any aqueous material, as for example, water (e.g. wastewater), saline or medium (e.g. cell growth medium) which is non-toxic to the microorganisms within. It will be appreciated that a mixture of saline or water or buffer with wastewater may be used or alternatively the particles may be gradually exposed to wastewater during activation thereof (e.g. by increasing the concentration of the wastewater within the saline). Alternatively, the microorganisms may be activated directly within the treating medium (e.g. wastewater). Thus, according to one embodiment, the microorganisms are activated in the same medium which needs to be treated (e.g. wastewater).

Following activation of the microorganisms, the particles may be relocated to a location of interest according to their intended use (e.g. into soil, wastewater etc.). It will be appreciated that the particles of the present invention may also be used without pre-activation.

The particles of the present invention are contemplated for varied uses, as described herein below.

According to one embodiment, there is provided a particle suitable for denitrification, comprising: (i) at least one inner core which comprises a solid matrix of

nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms, wherein the dried microorganisms are selected for facilitating de-nitrification; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water.

According to one embodiment, there is provided a particle suitable for purification of municipal wastewater, comprising: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms, wherein the dried microorganisms are selected for purification of municipal wastewater; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water.

According to one embodiment, there is provided a particle suitable for purification of food industry wastewater, comprising: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms, wherein the dried microorganisms are selected for purification of food industry wastewater; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water.

According to one embodiment, there is provided a particle suitable for purification of pharmaceutical wastewater, comprising: (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms, wherein the dried microorganisms are selected for purification of pharmaceutical wastewater; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water.

Thus, according to one aspect of the present invention, the particles are used for purifying water.

According to the present teachings, purifying water is effected by contacting the water with at least one particle under conditions that allow the microorganisms to decontaminate the water.

The temperature under which the decontamination procedure is carried out is selected such that it does not affect the viability of the microorganisms.

Use of the particles is effective for the length of time that the microorganisms remain viable and are capable of carrying out the decontamination procedure. Once the microorganisms are no longer effective, the particles may be removed and depending on the level of the contamination additional particles may be added.

According to an embodiment, the contacting is effected for a period of about 2-24 weeks, about 2-22 weeks, about 2-20 weeks, about 2-18 weeks, about 2-16 weeks, about 2-14 weeks, about 2-12 weeks, about 2-10 weeks, about 4-10 weeks, about 6-10 weeks, about 8-10 weeks, about 6-8 weeks, about 4-6 weeks, about 2-4 weeks, about 10-12 weeks, about 12-14 weeks, about 14-16 weeks, about 16-18 weeks, about 18-20 weeks, about 20-22 weeks, or about 22-24 weeks.

According to a specific embodiment, the contacting is effected for a period of about 2-60 weeks.

According to a specific embodiment, the contacting is effected for a period of about 2-45 weeks.

According to a specific embodiment, the contacting is effected for a period of about 2-24 weeks.

According to another specific embodiment, the contacting is effected for a period of about 6-8 weeks.

According to a specific embodiment, the contacting is effected for a period of about 8-12 weeks.

As used herein the phrase "purifying water", refers to the process of removing undesirable chemicals, materials, and biological contaminants from the water. Water purification may be designed for a variety of purposes, including for drinking or for meeting the requirements of medical, pharmacology, agriculture, chemical and industrial applications.

As used herein the phrase "decontaminate water" refers to the process of removal of poisonous or otherwise harmful substances, such as noxious chemicals, from the water.

Preferably, the water is decontaminated by at least about 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % or preferably by about 100 %.

It will be appreciated that any water source in need thereof may be purified according to the present teachings, including, but not limited to, drinking water, waste water (e.g. petroleum wastewater, heavy metal wastewater, municipal wastewater, industrial wastewater, agricultural wastewater, domestic wastewater, food industry wastewater, pharmaceutical industry wastewater), bathing water (e.g. pool, bath water), aquaculture water, or large water source (e.g. ocean, river, pond). The water may be fresh water or salt water.

As used herein, the term "wastewater" refers to any water that has been adversely affected in quality by human activity. Such wastewater can encompass a wide range of potential contaminants and concentrations thereof (specific examples are provide below). The wastewater may comprise treated wastewater which needs further purification (e.g. removal of contaminants e.g. nitrates or ammonia therefrom).

According to one aspect of the present invention, there is provided a method of purifying municipal wastewater, the method comprising contacting the municipal wastewater with a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater, under conditions that allow the microorganisms to decontaminate the municipal wastewater, thereby purifying the municipal wastewater.

As used herein, the term "municipal wastewater" refers to the subset of wastewater that is contaminated with feces or urine and also typically includes domestic, municipal or industrial liquid waste products disposed of, usually via a pipe or sewer or similar structure, sometimes in a cesspool emptier. Municipal wastewater is also referred to as sewage. Some components of municipal wastewater are listed in Table 1B above. Typically municipal wastewater contains organic matter, oil, fats, aromatic compounds, phosphorus, traces of drugs (e.g. antibiotics, NSAIDS-non-steroidal anti inflammatory drugs), nitrogen compounds and ammonia.

According to an exemplary embodiment, municipal wastewater treatment is typically carried out using particles comprising microorganisms selected for purification of municipal wastewater and microorganisms selected for purification of petroleum wastewater. Guidelines for selection of such microorganisms are provided in Tables 1A and 1B (hereinabove).

According to another aspect of the present invention, there is provided a method of purifying petroleum wastewater.

As used herein, the term "petroleum wastewater" relates to wastewater from e.g. petroleum refineries, chemical and petrochemical plants and includes a wide range of contaminants including, for example, heavy metals, organic compounds, petroleum hydrocarbons (short chains and long chains), solvents, pesticides, lead, polycyclic aromatic hydrocarbons - benzene, phenol, and toluene, Trichloroethylene (TCE), BETX.

According to yet another aspect of the present invention, there is provided a method of purifying food industry wastewater, the method comprising contacting the food industry wastewater with a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of food industry wastewater and a second population of dried microorganisms selected for purification of municipal wastewater, under conditions that allow the microorganisms to decontaminate the food industry wastewater, thereby purifying the food industry wastewater.

As used herein, the term "food industry wastewater" relates to wastewater generated from agricultural and food operations such as from dairy e.g. cheese, vegetable, fruit, and meat products. Food industry wastewater includes a wide range of contaminants including, for example, organic compounds, surfactants, pesticides, sugars, proteins, fats, oil, salts, anti-microbial agents (e.g. antibiotics, sulfides, phenols etc.), growth hormones, triglycerides, nitrogen compounds, natural hydrocarbons and ammonia.

According to an embodiment, the food industry wastewater comprises whey wastewater. Whey, as used herein, refers to the major by-product in the manufacture of cheese and typically comprises approximately 4.5 % (wt/vol) lactose, 0.8 % (wt/vol) protein, 1 % (wt/vol) salts, and 0.1 to 0.8 % (wt/vol) lactic acid.

According to yet another aspect of the present invention, there is provided a method of purifying pharmaceutical wastewater, the method comprising contacting the pharmaceutical wastewater with a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of pharmaceutical wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater, under conditions that allow the microorganisms to decontaminate the pharmaceutical wastewater, thereby purifying the pharmaceutical wastewater.

As used herein, the term "pharmaceutical wastewater" relates to wastewater generated from any process generating pharmaceuticals or personal care products including, but not limited to, prescription and over-the-counter human drugs, veterinary drugs, diagnostic agents, nutritional supplements, and other consumer products such as fragrances, cosmetics, and sun-screen agents. Pharmaceutical wastewater includes a wide range of contaminants including, for example, organic solvents, salts, aromatic compounds, phosphorus, traces of drugs, antimicrobial agents, glycerin, Absorbable Organic Halides (AOX) compounds, nitrogen compounds and ammonia.

Thus, any water contamination may be treated according to the present teachings including, but not limited to, the removal of chemicals including petroleum hydrocarbons, phosphorous compounds, nitrogen compounds, pesticides, sulfides, phosphates, cyanides, lead and other heavy metals, organic compounds including solvents (e.g. BTEX- benzene, toluene, ethylbenzene, xylenes and organic solvents), phenols, pharmaceutical mixture waste, detergents, organometallo constituents (e.g. vanadium and nickel), food industry by-products (e.g. whey), fats and oil (e.g. vegetable oil, olive oil, mineral oil, oil spills, floating oil, dispersed oil, dissolved oil).

Municipal enhancement treatment after wastewater treatment may also be carried out according to the present teachings. Thus, the particles may be used to degrade and accumulate difficult biodegradative organic matter and to reduce the concentration level of heavy metals, phosphorus, nitrogen and the like.

According to yet another aspect of the present invention, there is provided a method of reducing nitrate overload in water, the method comprising contacting the water with a particle comprising (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth; (ii) an inner membrane being fabricated

from a water-soluble polymer, the inner membrane surrounding the inner core and a population of dried microorganisms selected capable of facilitating de-nitrification of the water; and (iii) an outer porous membrane surrounding the inner membrane, the outer porous membrane being insoluble in water, thereby reducing nitrate overload in water.

Preferably, reducing nitrate overload in water is effected such that there is a reduction of at least about 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % or preferably by about 100 % of nitrate.

Typically reducing nitrate overload is effected by utilizing a de-nitrification process in which nitrate is converted to nitrogen gas (i.e. atmospheric nitrogen) and evaporates from the water.

According to one embodiment, the contacting is effected under anaerobic conditions.

According to another embodiment, the contacting is effected in the absence of non-particle associated organic matter (i.e. organic matter is not added directly to the water only within the particles of the invention).

According to another aspect of the invention, different types of particles may be used in parallel or subsequent to one another in order to increase purification processes. Thus, for example, particles for facilitating de-nitrification may be utilized along with particles for municipal wastewater treatment and/or with particles for petroleum wastewater treatment and/or with particles for pharmaceutical wastewater treatment and/or with particles for food industry wastewater treatment in order to increase water purification e.g. to reduce nitrate overload in water.

According to one embodiment, the method of reducing nitrate overload in water is further affected using particles suitable for oxygen enrichment.

According to one embodiment, there is provided a method of treating water, the method comprising contacting the water with a plurality of particles, wherein the plurality of particles comprise: (i) a first population of dried microorganisms selected for purification of municipal wastewater; (ii) a second population of dried microorganisms selected for purification of petroleum wastewater; and (iii) a third population of dried microorganisms selected for de-nitrification of water; under conditions that allow the microorganisms to purify the water, thereby treating the water.

According to one embodiment, (i) and (ii) are effected simultaneously or sequentially.

According to one embodiment, (i) and (ii) are effected under aerobic conditions.

According to one embodiment, (iii) is effected under anaerobic conditions.

According to one embodiment, the aerobic conditions comprise the presence of at least one particle suitable for oxygen enrichment.

According to one embodiment, (iii) is effected following (i) and (ii).

According to one embodiment, the method further comprises at least one particle suitable for oxygen enrichment.

According to one embodiment, contacting with (i) and (ii) and the particles suitable for oxygen enrichment are effected simultaneously.

The present teachings may be combined with any other water purifying methods including physical (e.g. filtration and sedimentation), chemical (e.g. flocculation and chlorination and the use of electromagnetic radiation such as ultraviolet light) or biological treatment processes (e.g. slow sand filters or activated sludge).

It will be appreciated that the particles of the present invention may be used to improve and/or stabilize water purifying treatments.

Thus, according to another aspect, there is provided a method of reducing sludge production during wastewater purification, the method comprising contacting the wastewater with a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater, under conditions that allow the microorganisms to decontaminate the wastewater, thereby reducing sludge production during the wastewater purification.

As used herein, the term "sludge" refers to the precipitated solid matter produced during wastewater purification processes (e.g. such as the type precipitated by municipal wastewater treatment, e.g. sewage treatment).

As used herein, the phrase "reducing sludge production" refers to the reduction of about 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % or 100 % in the amount of sludge in a bioreactor (or any other vessel used for wastewater purification) as compared to a bioreactor not comprising the particles of the present invention.

It will be appreciated that reducing sludge production is typically effected in any wastewater purification system. Thus, for example, reducing sludge production may be effected in petroleum wastewater, municipal wastewater, pharmaceutical wastewater, nitrogen enriched wastewater and food industry wastewater system (e.g. bioreactor).

According to a specific embodiment, reducing sludge production is effected in municipal wastewater treatment.

According to another aspect, there is provided a method of stabilizing a wastewater purification treatment, the method comprising contacting the wastewater with a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater, under conditions that allow the microorganisms to decontaminate the wastewater, thereby stabilizing the wastewater purification treatment.

As used herein, the phrase "stabilizing a wastewater purification treatment" refers to prevention of reduction in biodegradation performance, of overflow and/or of foaming of the wastewater purification system or treatment. Typically, addition of the particles of the present invention leads to a steadier purification process which efficiently purifies the water for a prolonged period with minimum overflow of the system (e.g. bioreactor). Typically, the purification treatment is more stable by about 10 %, 20 %, 30%, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % or 100 % as compared to a purification treatment not comprising the particles of the present invention.

Thus, the particles of the present invention may be incorporated with presently known systems as for example in physicochemical procedures of heavy metal treatment (Figures 17) and sour water strippers (Figures 18).

For example, in the physicochemical procedure [process flow draw (PFD) presented in Figure 17], the particles may be added to the reactor number 2 (T-2) in which sedimentation of heavy metal takes place. In order to confirm to the use of particles, the system may need to be adjusted, as for example, by the replacement of the reactor mixer with diffusers.

In refinery wastewater treatment (SWS - Sour Water Stripper, presented in Figure 18), the particles may be added to the bioreactor.

It will be appreciated that when the particles of the present invention are combined with other decontamination systems, different parameters including e.g. pH, chemicals, oxidizers, metals, coagulants (e.g. Aluminum sulfate, Aluminum Chloro Hydrate, Ferric chloride, Ferric/ferrous sulfate) and flocculants (e.g. FL-neg, FL-2, FL-pos) may be adjusted in the wastewater to enable optimal microorganism viability and activity.

The particles of the present invention can be targeted to a specific area. Thus, if for example a water surface needs to be decontaminated (e.g. for treatment of an oil spill or other floating hazardous substances) then the particles may be generated in a manner such that they float (e.g. may be generated without the addition of glass beads). Alternatively, if an area below the water surface needs to be decontaminated (e.g. petroleum or nitrogen, dissolved organic matter), then the particles may be generated such that they do not float thereby enabling them to target the contamination at particular depths below the water surface.

As mentioned above, the particle of the invention may be utilized in open spaces (e.g. under aerobic conditions) or within closed containers (e.g. under anaerobic conditions).

According to one embodiment, the particles may be utilized for aquaculture.

The present teachings further contemplate the use of the particles for treating soil contamination.

As used herein the term "soil contamination" refers to the presence of xenobiotic (man-made) chemicals or other alteration in the natural soil environment, such as organic compounds, metals and oils.

It will be appreciated that any soil contamination may be treated according to the present teachings including, but not limited to, rupture of underground storage tanks, application of pesticides, percolation of contaminated surface water to subsurface strata, oil and fuel dumping, leaching of wastes from landfills or direct discharge of industrial wastes to the soil. These include decontamination of chemicals including petroleum hydrocarbons, solvents, pesticides, lead and other heavy metals.

Preferably, the soil is decontaminated by at least about 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % or preferably by about 100 %.

It will be appreciated that prior to treating soil contamination, the particles are activated in a liquid substance (as described in detail above).

For both soil and water decontamination one type of particle (i.e. comprising identical microorganisms) may be used. Alternatively, two or more non-identical particles comprising different populations of microorganisms may be used (e.g. a first population of dried microorganisms and a second population of dried microorganism). These particles may be used concomitantly or subsequent to each other (e.g. at the same time or at different times as needed).

According to one embodiment, different ratios of such particles may be used. Thus, for example, a ratio between the first population of dried microorganisms and the second population of dried microorganisms may be of 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9 or 1:10. According to a specific embodiment, the ratio between the first population of dried microorganisms and the second population of dried microorganisms is 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 or 10:1.

According to another embodiment, the ratio between the first population of dried microorganisms and the second population of dried microorganisms is 6:1.

According to a specific embodiment, the ratio between the first population of dried microorganisms selected for purification of municipal wastewater and the second population of dried microorganisms selected for purification of petroleum wastewater for the treatment of municipal wastewater is 6:1.

According to a specific embodiment, the ratio between the first population of dried microorganisms selected for purification of municipal wastewater and the second population of dried microorganisms selected for purification of petroleum wastewater for reduction of sludge production or for stabilizing a wastewater purification treatment is 6:1.

According to a specific embodiment, the ratio between the first population of dried microorganisms selected for purification of food industry wastewater and the second population of dried microorganisms selected for purification of municipal wastewater for the treatment of food industry wastewater is 6:1.

According to another specific embodiment, the ratio between the first population of dried microorganisms selected for purification of pharmaceutical wastewater and the

second population of dried microorganisms selected for purification of petroleum wastewater for the treatment of pharmaceutical wastewater is 1:1.

If more than two populations of dried microorganisms are used, than the ratios may be adjusted as needed.

According to one embodiment, the ratio of the first population of dried microorganisms, the second population of dried microorganisms and the third population of dried microorganisms is 1:1:1, 1:1:2, 1:1:3, 1:1:4, 1:1:5, 1:1:6, 1:1:7, 1:1:8, 1:1:9, 1:1:10, 1:2:1, 1:3:1, 1:4:1, 1:5:1, 1:6:1, 1:7:1, 1:8:1, 1:9:1, 1:10:1, 2:1:1, 3:1:1, 4:1:1, 5:1:1, 6:1:1, 7:1:1, 8:1:1, 9:1:1, 10:1:1, 1:2:2, 1:3:3, 1:4:4, 1:5:5, 1:6:6, 1:7:7, 1:8:8, 1:9:9, 1:10:10, 2:2:1, 3:3:1, 4:4:1, 5:5:1, 6:6:1, 7:7:1, 8:8:1, 9:9:1 or 10:10:1. However, any combination may be used in accordance with the present teachings.

According to a specific embodiment, the ratio of the first population of dried microorganisms, the second population of dried microorganisms and the third population of dried microorganisms is 1:1:2.

According to one embodiment, the ratio of the particles is selected with respect to the type of water to be treated, the amount of water to be treated, the sizes of the host bioreactor, the retention time of the water within the treatment facility, and the contaminates type to be treated.

For example, the ratio of the particles to the water (e.g. wastewater) may be between about 0.01-20, between about 0.01-10, between about 0.01-5, between about 0.01-2, between about 0.01-0.1, between about 0.01-0.5, between about 0.01-1, between about 0.1-20, between about 0.1-10, between about 0.1-5, between about 0.1-2, between about 0.1-1, between about 1-20, between about 1-10, between about 1-5, between about 1-3, between about 1-2, between about 3-5, between about 5-10, between about 10-20 particles per cube water.

It will be appreciated that the ratio of the number of particles to water (e.g. wastewater) treatment volume can be determined by one of ordinary skill in the art in view of the present teachings and will typically take into account the type and the concentration of the contaminant (e.g. whey, petroleum, nitrate, ammonia, etc.).

Thus, for example, if the treatment tank (e.g. bioreactor) is 830 cube meter and the hydraulic retention time of the wastewater within the tank is approximately 6 hours (2400 cube meter per day treated by each bioreactor), then the number of particles may

range from about 100 particles up to about 10,000 particles, from about 500 particles up to about 9000 particles, from about 1000 particles up to about 8000 particles, from about 1500 particles up to about 6500 particles, from about 2500 particles up to about 5000 particles or from about 3000 particles up to about 4000 particles.

For soil and water decontamination, the particles may comprise microorganisms which have been isolated from the contaminated area. Thus, microorganisms (e.g. bacteria) may be obtained from the desired decontaminated area, isolated and dried prior to encapsulation thereof in the particles. The microorganism may be obtained from commercial companies as for example from USAbioproducts (Type 4, Type 2), Acron Biotechnical Corporation (ENSPOR S1), Advanced BioTech (Bioworld), One Biotechnology (BioOne®), Natural Environmental Systems, LLC (NE8000PH, NE2000MUN), A&V Envirotech (Bacti-Bio 1100G), BPC (BPC-ACT™), or Kazanci Environmental Technics (DC0003).

It will be appreciated that a single particle may comprise a single type of microorganism or alternatively may comprise different types of bacteria (e.g. different bacterial blends).

Determination of the microorganism population or populations to be used can be determined by one of ordinary skill in the art. An exemplary list of microorganisms and their possible applications is listed in Table 3, below.

**Table 3**

| Reference                                      | Reaction type        | Application                                                                                | Microorganisms                                                              |
|------------------------------------------------|----------------------|--------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| <b>Heavy metal absorption and accumulation</b> |                      |                                                                                            |                                                                             |
| Luque-Almagro et al.                           | Aerobic              | Alkaline metal-cyanid complex (nitrogen source)<br>Cyanid degradation and metal absorption | 1. <i>Pseudomonas fluroscens</i><br>2. <i>P. pseudoalcaligenes</i> CECT5344 |
| Laddaga, R.A., et al.                          |                      | Cadmium absorption via nonspecific cation transporter                                      | <i>Bacillus subtilis</i>                                                    |
|                                                | Aerobic or anaerobic | Metal (Cu <sup>+2</sup> ) fixation by bacterial cell wall                                  | <i>Bacillus licheniformis</i>                                               |

|                                        |                       |                                                                                                                                            |                                   |
|----------------------------------------|-----------------------|--------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------|
| Shuttleworth, K.L. and R.F. Unz        | Anoxic Or aerobic     | Metal uptake (nickel, zink and copper). Calcium and magnesium competed with zinc for binding sites. Lactate enhanced the uptake of nickel. | <i>Thiothrix</i> strain A1        |
| von Canstein, H., et al.,              | Aerobic               | Mercury uptake                                                                                                                             | <i>P. putida Spi3</i>             |
| <b>Phosphate degradation</b>           |                       |                                                                                                                                            |                                   |
| He, S. et al. and Ahn, J., et al.,     | Aerobic and anaerobic | Intercellular accumulation of phosphate in the form of poly(P)                                                                             | <i>Candidatus Accumulibacter</i>  |
| Kong, Y. et al.                        | Aerobic and anaerobic | Intercellular accumulation of phosphate in the form of poly(P)                                                                             | <i>Actinobacteria</i> sp.         |
| <b>Petroleum hydrocarbon degrading</b> |                       |                                                                                                                                            |                                   |
| Van Hamme, J.D. et al.                 | Aerobic               | Naphthalene and Salicylate                                                                                                                 | <i>P. putida Gp1</i>              |
| Van Hamme, J.D. et al.                 | Aerobic               | Naphthalene                                                                                                                                | <i>P. putida</i> NCIB9816         |
| Van Hamme, J.D. et al.                 | Aerobic               | Dibenzothiophene Naphthalene phenanthrene                                                                                                  | <i>Pseudomonas</i> sp. strain C18 |
| Van Hamme, J.D. et al.                 | Aerobic               | Naphthalene                                                                                                                                | <i>Pseudomonas</i> sp. Strain C18 |
| Van Hamme, J.D. et al.                 | Aerobic               | Naphthalene Phenanthrene<br>A variety of homo-hetro-and monocyclics converted to phenols                                                   | <i>Pseudomonas putida</i> OUS82   |
| Van Hamme, J.D. et al.                 | Aerobic               | Naphthalene 2-Methynaphthalene                                                                                                             | <i>Pseudomonas stutzeri</i> AN10  |

|                        |           |                                                                                                                        |                                                             |
|------------------------|-----------|------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------|
| Van Hamme, J.D. et al. | Aerobic   | Phenanthrene                                                                                                           | <i>Nocardioides</i> sp. Strain KP7                          |
| Van Hamme, J.D. et al. | Aerobic   | Naphthalene<br>Toluene<br>Indene                                                                                       | <i>Rhodococcus</i> sp. Strain 124                           |
| Van Hamme, J.D. et al. | Aerobic   | Anthracene<br>Phenanthrene<br>Fluoranthene<br>Pyrene, benzo [a]pyrene, 1-nitropyrene                                   | <i>Mycobacterium</i> sp. Strain PYR-1                       |
| Van Hamme, J.D. et al. | Aerobic   | Phenanthrene<br>Anthracene,<br>benzo[b]fluoranthene<br>Naphthalene<br>Fluoranthene, pyrene<br>Intermediate catabolites | <i>Sphingomonas paucimobilis</i> var EPA505                 |
| Van Hamme, J.D. et al. | Aerobic   | n-alkylbenzene<br>n-alkylcyclohexene                                                                                   | <i>Alcanivorax</i> sp. Strain MBIC 4326                     |
| Van Hamme, J.D. et al. | Aerobic   | Naphthalene phenanthrene                                                                                               | <i>Burkholderia</i> sp. RP007                               |
| Shen, H. and Y.T. Wang | Aerobic   | Using Chromium and phenol as a sole carbon source                                                                      | <i>P. putida</i> DMP-1 & <i>Escherichia coli</i> ATCC 33456 |
| Van Hamme, J.D. et al. | Anaerobic | Toluene                                                                                                                | <i>Blastochloris sulfovirens</i> ToP1                       |
| Van Hamme, J.D. et al. | Anaerobic | Ethylbenzene                                                                                                           | <i>Azoarcus</i> sp. Strain EB1                              |
| Van Hamme, J.D. et al. | Anaerobic | Toluene, <i>m</i> -xylene                                                                                              | <i>Azoarcus</i> sp. Strain T                                |
| Van Hamme, J.D. et al. | Anaerobic | Toluene, <i>m</i> -xylene                                                                                              | <i>Azoarcus</i> sp. Strain Td15                             |
| Van Hamme, J.D. et al. | Anaerobic | Toluene                                                                                                                | <i>Azoarcus</i> sp. Strain To14                             |
| Van Hamme, J.D. et al. | Anaerobic | Benzene, toluene                                                                                                       | <i>Dechloromonas</i> sp. Strain JJ                          |

|                        |           |                                          |                                     |
|------------------------|-----------|------------------------------------------|-------------------------------------|
| Van Hamme, J.D. et al. | Anaerobic | Benzene, toluene                         | <i>Dechloromonas sp.</i> Strain RCB |
| Van Hamme, J.D. et al. | Anaerobic | Naphthalene                              | <i>Pseudomonas sp.</i> Strain NAP-3 |
| Van Hamme, J.D. et al. | Anaerobic | Ethylbenzene, toluene                    | <i>Pseudomonas sp.</i> Strain EbN1  |
| Van Hamme, J.D. et al. | Anaerobic | C <sub>14</sub> -C <sub>20</sub> alkanes | <i>Pseudomonas sp.</i> Strain HdN1  |
| Van Hamme, J.D. et al. | Anaerobic | C <sub>6</sub> -C <sub>8</sub> alkanes   | <i>Pseudomonas sp.</i> Strain HxN1  |
| Van Hamme, J.D. et al. | Anaerobic | Toluene, <i>m</i> -xylene                | <i>Pseudomonas sp.</i> Strain M3    |
| Van Hamme, J.D. et al. | Anaerobic | Toluene, <i>m</i> -xylene                | <i>Pseudomonas sp.</i> Strain mXyN1 |
| Van Hamme, J.D. et al. | Anaerobic | C <sub>8</sub> -C <sub>12</sub> alkanes  | <i>Pseudomonas sp.</i> Strain mXyN1 |
| Van Hamme, J.D. et al. | Anaerobic | Toluene, <i>m</i> -xylene                | <i>Pseudomonas sp.</i> Strain PbN1  |

Table 3 references:

Luque-Almagro, V.M., et al., Appl Environ Microbiol, 2005. 71(2): 940-7  
 Laddaga, R.A., et al., J Bacteriol, 1985. 162(3): 1106-10  
 Shuttlesworth, K.L. and R.F. Unz, Appl Environ Microbiol, 1993. 59(5): 1274-1282  
 von Canstein, H., et al., Appl Environ Microbiol, 2002. 68(4): 1938-46  
 von Canstein, H., et al., Appl Environ Microbiol, 1999. 65(12): 5279-84  
 He, S. et al., Appl Environ Microbiol, 2007. 73(18): 5865-74  
 Ahn, J., et al., Appl Environ Microbiol, 2007. 73(7): 2257-70  
 Kong, Y. et al., Appl Environ Microbiol, 2005. 71(7): 4076-85  
 Van Hamme, J.D. et al., Microbiol Mol Biol Rev, 2003. 67(4): 503-49  
 Shen, H. and Y.T. Wang, Appl Environ Microbiol, 1995. 61(7): 2754-8

Thus, for example, municipal wastewater treatment is typically carried out using particles comprising microorganisms selected for purification of municipal wastewater and microorganisms selected for purification of petroleum wastewater. Such microorganisms can be selected by one of ordinary skill in the art in light of the present teachings and using, for example, the lists provided in Tables 1A, 1B and 3 (hereinabove).

Food industry wastewater treatment is typically carried out using particles comprising microorganisms selected for purification of food industry wastewater and microorganisms selected for purification of municipal wastewater. Such microorganisms can be selected by one of ordinary skill in the art in light of the present teachings and using, for example, the lists provided in Tables 1B, 1C and 3 (hereinabove).

Pharmaceutical wastewater treatment is typically carried out using particles comprising microorganisms selected for purification of pharmaceutical wastewater and microorganisms selected for purification of petroleum wastewater. Such microorganisms can be selected by one of ordinary skill in the art in light of the present teachings and using, for example, the lists provided in Tables 1A, 1D and 3 (hereinabove).

In order to enhance the microorganism activity in decontamination of soil or water, the inner cores of the particles may be formulated to be devoid of essential elements, such as e.g. nitrogen, specific hydrocarbons, iron, magnesium, phosphorus and sulfur, of amino acids, peptides, proteins, carbohydrates, sugars or carbon source such as glucose, of various salts such as NaCl and of other compounds such as lactate. Elimination of such elements from the inner core will compel the microorganisms to rely on elements from the contaminated area (e.g. soil or water).

According to one embodiment, there is provided an article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater.

According to another embodiment, there is provided an article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of food industry wastewater and a second population of dried microorganisms selected for purification of municipal wastewater.

According to another embodiment, there is provided an article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of pharmaceutical

wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater.

According to another embodiment, there is provided an article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater, a second population of dried microorganisms selected for purification of petroleum wastewater and a third population of dried microorganisms selected for de-nitrification of wastewater.

According to another embodiment, there is provided an article of manufacture comprising a plurality of particles, wherein the plurality of particles comprises a population of dried microorganisms selected for de-nitrification of wastewater.

According to one embodiment, the article of manufacture further comprises at least one particle suitable for oxygen enrichment.

As mentioned above, the particles of the present invention may be used for the generation of various molecules of interest such as biopolymers or recombinant polypeptides.

Thus, according to an aspect of the present invention, there is provided a method of synthesizing a molecule of interest by contacting a single or a plurality of particles with a liquid medium (e.g. in a bioreactor) under conditions (e.g. time and temperature) that allow synthesis of the molecule of interest and wherein the population of dried microorganisms within the particles is capable of synthesizing the molecule of interest on contact with the liquid medium.

As used herein the phrase "molecule of interest" refers to any molecule which is naturally or artificially synthesized by a microorganism.

According to one embodiment, the molecule of interest is a biopolymer.

According to one embodiment, the molecule of interest is a polypeptide.

As used herein the term "polypeptide" (or peptide) as used herein refers to a recombinant polypeptide or one which is naturally expressed (and preferably secreted) by the microorganisms.

Exemplary polypeptides include, but are not limited to, an antibody, an insulin, an interferon, a growth factor, a clotting factor, an enzyme, a diamine, a polyamine, an antibiotic, a glycopeptide, a lipopeptide, a hormone and a steroid.

According to another embodiment, the molecule of interest is an antibiotic.

Exemplary antibiotic agents which may be synthesized according to the present teachings include but are not limited to, penicillins (e.g. penicillin G, ampicillin and amoxicillin) cephalosporins (e.g. cephalexin, cefaclor and cefixime), carbapenems (e.g. meropenem and ertapenem), aminoglycosides (e.g. streptomycin, kanamycin, neomycin, tobramycin and gentamycin), macrolides (e.g. erythromycin, azithromycin and clarithromycin), lincosamides (e.g. clindamycin), streptogramins (e.g. quinupristin and dalbapristin), fluoroquinolones (e.g. ciprofloxacin, levofloxacin and norfloxacin), lincomycins, tetracyclines (e.g. chlortetracycline, oxytetracycline and doxycycline), chloramphenicol, griseofulvin, rifampin, mupirocin, cycloserine, polymyxine and aminocyclitols.

The microorganisms of the present invention may be genetically modified such that they may synthesize the desired molecules (e.g. hormone or antibiotic). Thus, for example, the microorganism may be genetically modified to express an enzyme or several enzymes which allows for the production of the hormone or antibiotic.

According to another embodiment, the microorganism may be modified to secrete an enzyme which migrates out of the particle into the medium where it catalyzes the production of a target agent (e.g. an antibiotic).

Any method known to one of ordinary skill in the art for genetic modification of an organism may be used according to the present teachings. Such methods include recombinant DNA technology as described for example in Studier et al. (1990) *Methods in Enzymol.* 185:60-89 and in U.S. Pat. Application No: 5,932,447.

The recombinant polypeptides and other contemplated molecules may be generated in vitro in mass production (e.g. in bioreactors) or in small quantities or home use (e.g. in small containers). For mass production, the particles are placed in a liquid medium (e.g. culture medium) preferably under sterile conditions; the polypeptides are secreted into the liquid medium and are purified prior to administration to the subject.

The present invention contemplates generating more than one molecule (e.g. recombinant polypeptide) in a single bioreactor. In this case, the microorganisms in each particle synthesize only one type of molecule. According to one embodiment, the particle is fabricated such that the molecule is not capable of exiting through the outer membrane into the medium – so as to avoid the different molecules (e.g. recombinant

polypeptides) mixing in the bioreactor. According to another embodiment, the molecule (e.g. recombinant polypeptide) is not secreted by the microorganisms, such that it is maintained in the particle (i.e. does not exit the particle). In order to distinguish between the different polypeptides, the particles may, for example, be labeled (as depicted in Figure 19A) as for example with a detectable moiety such as a radioisotope, a fluorescent or chemiluminescent compound or a tag. Alternatively, the particles may be cultured in a separate area in the bioreactor system (e.g. in divided internal chambers of a bioreactor, see Figure 19B).

Separation and purification of the molecules from the liquid medium may be carried out using any method known to one of ordinary skill in the art as for example by high-performance liquid chromatography (HPLC), normal phase HPLC (NP-HPLC), reversed phase HPLC (RPC) or size exclusion chromatography (SEC), based on their idiosyncratic polarities and interactions with the column's stationary phase (e.g. hydrophobic saturated carbon chains). Preferably the molecules are purified under sterile conditions. As mentioned above, the particles of the present invention may also be beneficial for generating molecules (e.g. polypeptides) with short half-lives. Such particles may be valuable when only small amounts are required e.g. for home or clinic uses. The particles may be placed in a small bioreactor (e.g. glass, bottle) with liquid medium. Following a finite amount of time (e.g. overnight), the liquid medium comprises the molecules of interest (since they have been secreted into the liquid medium by the microorganisms). The liquid medium may be administered to the subject without any intermediate steps (e.g. topically). The particles may be used for several weeks (e.g. 3-4 weeks) while the liquid medium may be used and replaced (e.g. with a fresh medium).

Alternatively, the molecules may be generated in vivo. In such cases, the particles are administered to the subject and synthesis of the molecules occurs inside the body. The molecules may be directly secreted from the particle to the diseased area, as explained herein below.

Accordingly, the present invention contemplates the use of the particles for treating medical disorders, such as gastrointestinal disorders, in a subject in need thereof.

As used herein the term "treating" refers to preventing, curing, reversing, attenuating, alleviating, minimizing, suppressing or halting the deleterious effects of the disease e.g. gastrointestinal disease. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a pathology, and similarly, various methodologies and assays may be used to assess the reduction, remission or regression of a pathology.

As used herein the phrase "a subject in need thereof" refers to a mammal, preferably a human subject, male or female of any age, who has been diagnosed with probable or definite gastrointestinal disease, e.g., a subject who experienced inflammatory colon disease. The diagnosis of a gastrointestinal disease may include any diagnosis test as, for example, laboratory tests, endoscopic evaluation, biopsies of the mucosa (e.g. for ulcerative colitis), barium follow-through x-ray (e.g. for Crohn's disease), and CT or MRI scans (e.g. for Crohn's disease).

As used herein the term "gastrointestinal disorder" refers to any disease that pertains to the gastrointestinal tract, also referred to as digestive diseases. These include diseases of the esophagus, stomach, first, second and third part of the duodenum, jejunum, ileum, the ileo-cecal complex, large intestine (ascending, transverse and descending colon) sigmoid colon and rectum.

Examples of such diseases include, but are not limited to, gastrointestinal tumors, inflammatory diseases, chronic inflammatory intestinal diseases, gastrointestinal diseases, autoimmune diseases of the gastrointestinal tract, intestinal diseases, chronic inflammatory intestinal diseases (Garcia Herola A. *et al.*, Gastroenterol Hepatol. 2000 Jan;23 (1):16), chronic inflammatory intestinal disease (Garcia Herola A. *et al.*, Gastroenterol Hepatol. 2000 Jan; 23 (1):16), celiac disease (Landau YE. and Shoenfeld Y. Harefuah 2000 Jan 16; 138 (2):122), colitis, ileitis and Crohn's disease.

It will be appreciated that for treatment of a disorder the microorganisms within the particles of the present invention are selected as those capable of producing and secreting an agent (e.g. a polypeptide, an antibiotic) such as an agent useful for the treatment of a gastrointestinal disorder.

Thus, the particles of the present invention can be administered to an organism per se, or in a pharmaceutical composition where it is mixed with suitable carriers or excipients.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the particles accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, intraperitoneal, intranasal, or intraocular injections.

According to a specific embodiment of the present invention, the particles are administered orally.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

It will be appreciated that the particles of the present invention may be coated with a soluble material (e.g. for pH control such as eudragit polymer) to allow molecule release in a specific area in the gastrointestinal tract.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be

suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

The pharmaceutical composition of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients (i.e. particles) effective to prevent, alleviate or ameliorate symptoms of a disorder (e.g., gastrointestinal disease) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The

dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to levels of the active ingredient which are sufficient to effectively regulate gastrointestinal disease treatment. Dosages necessary to achieve the desired effect will depend on individual characteristics and route of administration. Detection assays can be used to determine disease manifestation.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc. The dosage and timing of administration will be responsive to a careful and continuous monitoring of the individual changing condition.

Models for gastrointestinal diseases include e.g. animal models for inflammatory colon diseases such as of ulcerative colitis including trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats and mice [Komori et al., J Gastroenterol (2005) 40: 591–599].

According to the present invention at least one particle is administered to the subject to treat the gastrointestinal disorder. However, several particles may be administered as necessary. The particles may be administered concomitantly, or separately such as on the same day, or on different days, weeks or months as necessary.

Regardless of the above, the particle is administered at an amount selected to avoid unwanted side-effects.

It will be appreciated that the particles of the present invention are preferably secreted via the subject's stool following a few hours to several days of administration thereof.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

Another possible application of the particles of the present invention is for cosmetic applications. Thus, the particles of the present invention can be used as a supplement in a variety of cosmetics, as for example, to secrete substance (e.g. polypeptides) which may be used as is or added to cosmetics.

Cosmetics are substances used to enhance or protect the appearance or odor of the human body. Examples of cosmetics include skin-care creams, lotions, powders, perfumes, lipsticks, fingernail and toe nail polish, eye and facial makeup, perfumes, aftershaves, manicures, permanent waves, shaving foams and creams, hair colors, hair sprays and gels, deodorants, baby products, bath oils, bubble baths, bath salts, butters and many other types of products.

As used herein the term “about” refers to  $\pm 10\%$ .

The terms "comprises", "comprising", "includes", "including", “having” and their conjugates mean "including but not limited to".

The term “consisting of means “including and limited to”.

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the

additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described

embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

### EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture"

Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

## **EXAMPLE 1**

### ***Particle manufacture***

#### **MATERIALS AND EXPERIMENTAL PROCEDURES**

##### ***Freeze-dried microorganism (bacteria) manufacture***

Inventors used a freeze-dry method to produce a dry bacteria/microorganism powder according to previously described protocols [Leslie, S.B., et al., Appl Environ Microbiol (1995) 61(10): 3592-7; Sinskey, T.J. and G.J. Silverman, J Bacteriol (1970) 101(2): 429-37; Morgan, C.A., et al., J Microbiol Methods (2006) 66(2): 183-93; Costa, E., et al., J Appl Microbiol (2002) 92(5): 873-8]. The following steps were carried out:

- 1) Bacteria (E. coli TG1, E.coli TG1 pChv1, or E. coli DH5 $\alpha$ ) were grown overnight at 37 °C, 200 rpm, 18 h, in 1 L growth medium comprising 0.5 % yeast extract, 1 % tryptone, 0.5 % NaCl, and 5 % sucrose (as a protecting freeze agent).
- 2) The culture was centrifuged (10 min, 7000 g, 4 °C).
- 3) The culture was suspended in 8 ml ice cold phosphate buffer solution (PBS, Sigma).
- 4) The suspended culture was centrifuged (10 min, 7000 g, 4 °C).
- 5) The culture was suspended in 8 ml ice cold PBS comprising 5 % sucrose.
- 6) Steps 4-5 were repeated.
- 7) The culture was incubated at room temperature (22 °C) for 20 minutes, and was then divided into 1 ml doses for future viability counts (saving the precise volume).

- 8) The culture was incubated for 48-72 h at -80 °C.
- 9) The culture was freeze-dried for 52 h (1 Pa, -45 °C, 10 µHg).
- 9) The dried culture was then stored at room temperature inside a dissector.

#### ***Inner core design and manufacture***

The inner cores were made of LA (Luria Agar - 0.5 % yeast extract, 1 % tryptone, 0.5 % NaCl and 0.185 % agar) which were cast into 96 wells plates. After the polymerization of the LA, the inner cores were released from the plates onto petri dishes for drying in a biological hood for 72 hours. Next, the inner cores were sterilized using U.V radiation within the biological hood for several hours.

#### ***Other inner particle components***

Glass beads (1 mm diameter) were added to the particles to increase the weight of the particle and to provide additional surface area for biofilm formation.

#### ***The particle construct***

Gelatin capsules, size 000, were used to integrate all the inner components (microorganisms, inner core and glass beads) and to provide the foundation to built the outer membrane of the particle.

#### ***Constructing the outer membrane***

Two types of polymers were used to construct the outer membrane, ethyl cellulose and cellulose acetate as follows:

##### **Ethyl Cellulose (EC) outer membrane coating**

Ethyl cellulose polymer solution was prepared using a mix of two solvents, methanol and acetone, along with a plasticizer such as castor oil.

The protocol of the polymer solution:

Solution solvents (92 %): 80 % acetone and 20 % methanol.

Suspended solids (8 %): castor oil (12 %) and ethyl cellulose (88 %) (TAIAN RUITI CELLULOSE LTD., China).

The prepared solution was mixed with a stirrer for at least half an hour. Next, the inventor used a spray to coat the gelatin capsules with the polymer. The particles were put on an upside down 96-well plate and sprayed 4-5 times on each side. The polymer solution volume was calculated according the required thickness of the particle (determined according to the required resistance for a cutting force for a given time). The inventor used 13 ml or 15 ml of the polymer solution for coating of one particle. For

ethyl cellulose, 6 % and 8 % suspended solids were used within the polymer solution and the stability of both formulations was tested.

#### Cellulose Acetate outer membrane coating

The cellulose acetate polymer solution was prepared using a mix of two solvents, methanol and acetone, along with a plasticizer such as castor oil.

The protocol of the polymer solution:

Solution solvents (92 %): 80 % acetone and 20 % methanol.

Suspended solids (8 %): castor oil (12 %) and cellulose acetate (88 %) (EASTMAN, Switzerland).

The prepared solution was mixed with a stirrer for at least half an hour. Next, the inventor used a spray to coat the gelatin capsule with the polymer. The particles were put on an upside down 96-well plate and sprayed 4-5 times on each side. Each particle was coated with 8 ml of the polymer solution.

### **RESULTS**

The particle generated according to the present teachings comprised the following physical components: the outer membrane, the inner core and the microorganisms. Each component was prepared separately (except for the outer membrane). All of the inner components: the inner core, the glass beads and the microorganisms (e.g. bacteria) were integrated inside a gelatin capsule (size 000) and the outer membrane was constructed on the surface of the gelatin capsule (as described in detail in the Materials and Experimental procedures section above). Figure 1B presents an example of a particle prototype generated according to the present invention.

#### Manufacture of freeze-dried bacteria

Since the product of the present teachings was designed to have a long life span, the inventor used freeze-dried bacteria/microorganism which is known to be a preferred method for transporting and storing cultures of microorganisms. Figure 2 presents the viability at different time intervals (weeks) after rehydration. As shown in Figure 2, the dried culture showed stability for at least 5 weeks after rehydration. Moreover, the bacterial viability levels were kept above 7,500,000,000 bacteria per milliliter which is a preferred concentration for processing and for reducing culture contamination. The weight of 1 ml freeze-dried bacteria culture was 0.07 gr. The freeze-dried culture texture was observed as illustrated in Figure 3.

#### Inner core design and manufacture

The aim of the inner core inside the particle was to provide feeding nutrients to the culture and to provide additional biofilm formation surface area. LA (Luria Agar), a rich nutrient supplier solid agent for bacterial growth and prosperity and a surface area for biofilm formation, was used to generate the inner cores. Figure 4A shows the inner cores after polymerization (on left) and in comparison to a dry core (on right). Figure 4B depicts a lateral view of the inner core after polymerization and after sterilization.

#### Other Inner particle components

The rate of activation of the particle of the present invention depended on the penetration rate of water molecules into the particle. In order to prevent the particles from floating on the surface of the medium (which may cause delayed activation and loss of particle surface area – reduce the contact area with the medium), and to provide additional surface area for biofilm formation, inventors added glass beads to the particles.

#### The particle construct

The inventor of the present invention used a gelatin capsule (size 000) to integrate all of the inner components (microorganisms, inner core and glass beads) and to provide the foundations on which to build the outer membrane (see Figure 5).

#### Constructing the outer membrane

The inventor of the present invention used two types of polymers to construct the outer membrane, ethyl cellulose and cellulose acetate, and hence, constructed two types of particle prototypes. Ethyl cellulose or cellulose acetate, water insoluble porous polymers, were prepared as described in detail in the Materials and Experimental procedures section above. The particles were sprayed with ethyl cellulose or cellulose acetate by placing the particles on an upside down 96 wells plate and spraying each side 4-5 times with the desirable polymer (as shown in Figure 6). As depicted in Figure 7, particles coated with 8 % cellulose acetate displayed 1-3 weeks of stable coating.

**EXAMPLE 2*****Particle activation and biocompatibility*****MATERIALS AND EXPERIMENTAL PROCEDURES*****Particle manufacture***

Particles were prepared as described in Example 1, hereinabove.

***Particle activation and biocompatibility***

In order to activate the particles, the ethyl cellulose or cellulose acetate coated particles were placed in bottles containing PBS or Saline for different time periods.

Water content inside the particles was measured as a means to evaluate water flow rate. Particles weights were measured using a scale at different time intervals and estimation of the particle water content was calculated as percentage of the maximum weight of a filled water particle (the weight of a particle after the particle was filled with water).

Bacteria viability was measured by first collecting a sample of the liquid inside the particle using a syringe and needle. The sample supernatant was measured and diluted in saline (0.9 ml of saline was added to 0.1 ml sample) to produce culture viability counts in a LA nutrient plate. The inner core was analyzed for morphology and for viability. The solid phase of the particle which included the glass beads and the inner cores, were kept in an eppendorf tube and suspended in 1 ml of saline and vortexed for 30 seconds to remove all of the bacteria therefrom. Viable count was then performed on the supernatant as described above.

**RESULTS**

Since the biological process of the present invention can only be activated when the bacteria is transferred from a dry state to a liquid suspended state (planktonic state), liquid flow into the particle is an essential step. The goal of the present invention was to ensure that particle activation time was no more than several hours. In order to evaluate the particle activation time, the present inventor measured the weight of the particle at particular time intervals and estimated the particle water content as percentage of the maximum weight of a filled water particle (the weight of a particle after the particle was filled with water, the percentage of particle water content of the maximum water content – 1.4 gr ). Analysis of the results illustrated that 30 % of the particle water content was

the point of particle activation, as was demonstrated on a particle that was coated with 8 % ethyl cellulose.

Figure 8 summarizes the comparison results of the water flow rate between 15 ml and 13 ml coated 8 % ethyl cellulose particles. As shown in Figure 8, the present results indicated that ethyl cellulose 8 % (15 ml) coated particles had a faster particle activation rate. Particle activation of 15 ml coated Ethyl cellulose membrane (8 %) was achieved after 72 h as compared to 96 h for 13 ml coated particles. Moreover, particles that were coated in 15 ml of the polymeric solution reached their maximum weight (106 %) while particles coated with 13 ml reached only to 87 % of the maximum particle weight. Thus, the maximum particle weight was 2.04 gr and the maximum water content within the particle was 1.4 gr water. Non-activated particle weight was 0.64 gr (for 15 ml coated capsules) or 0.41 gr (for 13 ml coated capsules). After 4 weeks, the ethyl cellulose coated particles were slightly expended (Figure 7A). Particles devoid of bacteria showed a significant inhibition in water penetration. These results suggest that the bacteria protective agent (a sugar) used for freeze-drying generated an osmotic pressure which accelerated the water flow rate into the particle.

Cellulose Acetate membranes (8 %) were examined to estimate the activation time point. The present inventor examined particles coated with 8 ml cellulose acetate (8 %), filled with different types of bacteria: E.coli TG1 and E.coli TG1 pChv1. As shown in Figure 9, the bacteria type had no influence on water flow into the particles. The activation time point of both of the cellulose acetate membrane particles was between 48-72 h.

Comparison of water flow rate between ethyl cellulose and cellulose acetate particles (Figure 10) illustrates a slightly faster rate of particle activation in cellulose acetate coated particles.

The main goal of this experiment was to evaluate the biocompatibility of the bacterial culture inside the particle, which effects both the product life span prior to and following activation. In order to assess biocompatibility, the inventor placed the different particles in saline (see Figures 11A-C). The particles were maintained in the saline for 5 weeks and the liquid inside the particles was checked on a weekly basis by collection using a syringe and needle. The collected sample volume was then measured and diluted in saline to produce culture viability counts in LA nutrient plate. The inner

core was assessed for both morphology and for viability. The glass beads and the inner cores were kept in an eppendorf tube and suspended in 1 ml of saline and vortexed for 30 seconds to remove all of the bacteria therefrom prior to viability counts. Viability was measured in the supernatant as described above.

Viability of the inner core typically showed reduction of one log compared to the liquid viability (data are not shown). This indicated that inner cores (two in one particle) were crucial for the stability of the culture inside the particle and had a great contribution to the overall bacteria counts inside the particle. The liquid (planktonic) bacteria population (after particle activation) could originate from several sources: A. Starter - the inserted freeze-dried culture, B. Planktonic bacteria reproduction, or C. Inner core bacteria migration to the liquid phase.

The bacteria viability within particles (i.e. not in the solid phase of the particle) of two culture productions which were coated with cellulose acetate (8 %) are depicted in Figure 12. As shown in Figure 12, after 5 weeks of particle incubation in saline, the bacteria culture remained stable at an average concentration of  $8 \log_{10}$  CFU/ml. This concentration was considered high and provided sufficient biological process. Furthermore, these high bacterial levels could eliminate possible contamination of the particle (by other microorganisms) as the bacteria occupy the majority of the surface area of the inner core (i.e. the only food source within the particle which was incubated in saline). Thus, the cellulose acetate particle presented good biocompatibility for at least 5 weeks after activation.

Ethyl cellulose-coated particles were also tested for viability. Figure 13 shows the viability of an *E. coli* TG1 culture within these particles at different time intervals. A typical bacterial growth curve was observed by the bacterial culture within the particle. Thus, in the first week an environment culture adaptation of the bacterial culture was observed which was followed by logarithmic phase for more than 1 week. The decrease in the curve (from 2 weeks to 4 weeks) presented a withdrawal in the bacteria concentration number. After 4 weeks, the culture concentration was 10,000,000 bacteria per 1 ml liquid. This bacterial concentration is sufficient to provide a desirable biological process.

Thus, both the cellulose acetate and ethyl cellulose coated particles presented good bacterial biocompatibility for at least 4 weeks of incubation in saline.

**EXAMPLE 3*****Macromolecules trafficking validation*****MATERIALS AND EXPERIMENTAL PROCEDURES*****Preparation bacteria***

A bacterial culture (E. coli TG1) harboring a molecular marker was used to evaluate molecule trafficking across the particle membrane. The molecular marker used was a plasmid (pChv1 - 13 kbp) that contained the entire luciferase system derived from *Vibrio fischeri*. In the presence of the system inducer (Homo serine lactone - C10 hydrocarbon, see Figure 14), the plasmid is activated and transcription and translation of the luciferase enzyme is accomplished. Luciferase catalyzes the metabolic reaction of transferring fatty acids into aldehydes. The byproduct of this reaction is light emission (480 nm) which may be measured by a spectrophotometer.

***The membrane permeability validation system***

Two systems were used: 1) the experimental system in which the inducer (1 µg/ml) was added to the particle medium (saline), and 2) the control system which did not contain the inducer in the particle medium (as shown in Figure 15). The particles were incubated inside each of the mediums for one hour prior to extraction of the particle inner medium (containing the planktonic bacterial culture) using a syringe with a needle. The sample was read in a Bio-Tek spectrophotometer (light detector sensitivity 125). Light emission was measured in the test culture, the control system and a blank medium (saline).

**RESULTS**

Light emission was measured for the experimental system which contained the inducer, the control system and a blank medium (saline). Significant light emission of the test system represented penetration of the inducer and gene activation of the molecular luciferase system within the bacterial culture. Table 4, below, shows the light emission results obtained. The results indicate that the inducer penetrated the particles in less than 40 minutes (measured from the addition of the inducer). It may be estimated that it takes at least 20 minutes to activate the molecular marker. After one hour of incubation, light emission in the experimental system (comprising the inducer) was

higher by more than 7 times in comparison to the control system which did not contain the inducer in the particle medium.

It may be concluded that cellulose acetate membranes comprise a porosity which allow molecules of the size of the inducer to be transported from the medium to the particle and back. Furthermore, the bacterial culture within the particle remained stable for at least five weeks.

**Table 4: Results of light emission**

| <b>Luminescence ratio (%)</b> | <b>Sample #2</b> | <b>Sample #1</b> | <b>Control #2</b> | <b>Control #1</b> | <b>Blank #2 (saline)</b> | <b>Blank #1 (saline)</b> |
|-------------------------------|------------------|------------------|-------------------|-------------------|--------------------------|--------------------------|
| 308%                          | 1935             | 2037             | 615               | 673               | 597                      | 559                      |
| 307%                          | 1967             | 2088             | 664               | 659               | 592                      | 533                      |
| 340%                          | 2135             | 2044             | 652               | 584               | 511                      | 521                      |
| 361%                          | 2192             | 2193             | 558               | 658               | 524                      | 568                      |
| 509%                          | 2659             | 2712             | 562               | 493               | 537                      | 496                      |
| 463%                          | 2758             | 2634             | 572               | 592               | 563                      | 493                      |
| 479%                          | 3150             | 3100             | 666               | 638               | 519                      | 573                      |
| 731%                          | 5190             | 5191             | 723               | 696               | 602                      | *582                     |

Light emission results of the test system, the control system and blank (saline). After half an hour the ratio between light emission of the test group and the control group was 7 times higher.  
\*The last reading was made after half an hour since the first read

## **EXAMPLE 4**

### ***Petroleum wastewater treatment indication***

#### **MATERIALS AND EXPERIMENTAL PROCEDURES**

##### ***Product manufacture***

A particle, suitable for petroleum wastewater treatment, was manufactured under semi-sterile conditions (designated herein as NatiCap<sub>petroleum</sub>) as follows: 1) The inner core components included: 3 glass beads, 2 dry inner cores (nutrient agar, LA), a bacterial blend, a commercial microbial blend (USAbioproducs – biotreat type 4) which included selected adapted high potency microbes for biological biodegradation of petroleum hydrocarbons, and additional components including activated Carbon (dusk form) and degradation enzymes. All of the inner core components were inserted into a water-soluble gelatin capsule.

2) The gelatin capsule comprising all of the inner core components was coated with cellulose acetate 8 % polymer (15 ml per particle) as described in detail in Example 1, hereinabove.

### ***NatiCap<sup>TM</sup> petroleum Activation***

In order to activate the product, 75 particles of the NatiCap<sup>TM</sup> petroleum were placed in a 1 L container containing 500 ml of saline. The particles were incubated for 24 h prior to the experiment (after 24 h of activation the average weight of the particle was 1.88 gr, while the average dry particle weight was 1.067 gr - 58 % of water content).

### ***The model test***

The wastewater efficacy test was carried out in a bioreactor which was designed as follow:

The bioreactor was made of PVC: 40 cm X 20 cm X 25 cm. Two air pipes located 5 cm above the bottom of the bioreactor were spread lengthwise and contained air diffusers (one air diffuser every 5 cm). Both air pipes shared a common entry into the bioreactor. The air flow rate thru the air pipes was 550 liters per hour (using an air pump, Sere 550). The purpose of using air diffusers inside the bioreactor was to achieve good oxygen solubility in the wastewater and to generate fluid circulation. The test Process Flow Diagram (PFD) as used is depicted in Figures 16A-C. Sampling was completed through a valve at 12.5 cm (measured from the bottom of the reactor).

The wastewater tested was obtained from a refinery (Haifa, Israel). Prior to testing, the wastewater was treated with an API (water/oil separator) and DGF (Dissolved Gas Flotation) which decreases the emulsion and suspends solids inside the wastewater.

### ***Methods***

75 NatiCap<sup>TM</sup> petroleum particles were activated in 500 ml of saline and added to the bioreactor which contained 15 liters of the petroleum wastewater. Thus, the total fluid volume in the first cycle of the bioreactor was 15.5 liters. The particle/volume ratio was 1:200 ml. Prior to the addition of the particles, the wastewater was sampled.

Conditions in the bioreactor were cycled as follows: 72 hours of aerobic reaction conditions (air diffusers – 550 liters per minute) followed by 72 hours of anoxic conditions (without activating the air diffusers). For each new cycle (after 144 hours)

the treated wastewater was replaced with fresh petroleum wastewater (from the same source). The test experiment included 3 cycles (for a total of 18 days).

Sampling points:

1. Prior to the experiment (referenced for each cycle).
2. After 72 hours (aerobic stage).
3. After 144 hours (at the end of the anoxic stage).

Prior to wastewater sampling and the cycling waste exchange, the wastewater was air vortexed for 0.5 hour.

Bacterial viability was tested by adding the particle sludge into a vial (estimated volume of 1 ml) and 0.5 ml of saline. Serial dilutions with saline were performed and seeded on a nutrient agar (LA) using the Derigalski method [M.E. Madigan, J.M. Martinko, J. Parker, Microorganisms, 12th ed. (2000) Prentice Hall, Upper Saddle River, New Jersey].

## RESULTS

### Laboratory chemical analysis of the wastewater

COD (chemical oxygen demand) was measured as an indirect indicator for PTH (petroleum hydrocarbon) concentration reduction.

Cycle 1: At the end of the first cycle (144 h after incubation of the active particles in the wastewater), the present inventor observed only one broken particle and 16 of the particles were floating. One of the floating particles was removed for fluid content analysis of the bacterial composition (*Pseudomonas* Vs. *Bacillus*) and for inner core analysis. A black sludge (the active carbon and microorganisms mix) was observed inside the particle. The inner cores tested were wet, indicating massive fluid penetration.

Bacterial viability performed after 144 hours of treatment showed a bacterial concentration of about  $10^9$  -  $10^{12}$  CFU/ml. The bacterial mix culture contained at least 5 population types (microorganism population diversity). Table 5 (below) presents the results of the bacterial viability of a NatiCap<sup>TM</sup> petroleum particle. Moreover, the wastewater (medium) became cloudier indicating the presence of planktonic bacteria. After centrifugation (1.5 minutes, 13,000 rpm) of 1 ml of the treated wastewater, a 0.5 mm white pellet was obtained and the medium was clear, indicating significant presence of planktonic microorganisms (natural wastewater flora). Thus, it may be postulated that during the incubation time, particularly at the anoxic stage, a rich bacterium population

developed (within the medium), probably due to a viable organic Carbon source derived from the breakdown of the petroleum hydrocarbons by the particles. This result indicates that the particle effects the PTH by chapping the molecules into smaller hydrocarbon chains so as to generate a viable Carbon source for the natural wastewater flora. Accordingly, the chemical analysis results (after 3 days, at the end of the aerobic stage) indicate a significant reduction in the concentration of COD (chemical oxygen demand- from 335 mg/l to 270 mg/l, data not shown), indicating possibly reduction in PTH concentrations. Moreover, the following analysis tests performed exhibited an increase in biomass within the wastewater (planktonic microorganisms outside the particles): BOD (biological oxygen demand) test showed a shift from 60 mg/l to 105 mg/l (data not shown) and TSS (total suspended solids) showed a shift from 10 mg/l to 20 mg/l (data not shown). After the anoxic stage (at the end of the 6 day treatment), the chemical analysis of the wastewater indicate a significant increase in COD values. These results indicate that the anoxic stage at this point was not due to the treatment.

Cycle 2: The chemical analysis results (after 3 days - at the end of the aerobic stage) indicate a reduction in COD concentrations (from 335 mg/l to 300 mg/l, data not shown), indicating a possible reduction in PTH concentrations. As shown for the first cycle, the anoxic treatment stage did not contribute to the treatment and the COD results became much higher (up to 685 mg/l, data not shown).

Cycle 3: The chemical analysis results (after 3 days - at the end of the aerobic stage) indicate a significant reduction in the concentration of COD (from 335 mg/l to 225 mg/l, data not shown), indicating a possible reduction in PTH concentrations. As shown for the first cycle, the anoxic treatment stage did not contribute to the treatment and the COD results became much higher (up to 385 mg/l, data not shown).

#### Bacterial viability within the particle

After one cycle of the experiment (144 h after incubation of the active particles in the wastewater), one particle was taken and examined in order to estimate the bacterial colony forming units and the bacterial population diversity.

**Table 5: Bacterial viability**

| Discretion of the colonies (microorganisms population diversity)                            | Number of bacteria colony                                    | Dilution   |
|---------------------------------------------------------------------------------------------|--------------------------------------------------------------|------------|
| 5 (big white colonies, Yellow, white small colonies, white with clear end colonies, fungi). | $5 \times 10^9$ (cfu/ml)                                     | $10^{-6}$  |
| 4 (white, Yellow, white small colonies, white with clear end colonies).                     | $4 \times 10^{10}$ (cfu/ml)<br>$3.2 \times 10^{10}$ (cfu/ml) | $10^{-7}$  |
| 3 (white, Yellow, white small colonies)                                                     | $1.8 \times 10^{11}$ (cfu/ml)                                | $10^{-8}$  |
| 3 (white, Yellow, white small colonies)                                                     | $5 \times 10^{11}$ (cfu/ml)                                  | $10^{-9}$  |
| 3 (white, Yellow, white small colonies)                                                     | $6 \times 10^{12}$ (cfu/ml)<br>$3 \times 10^{12}$ (cfu/ml)   | $10^{-10}$ |

As depicted in Table 5, above, the bacterial viability within the particle indicate very good microorganism prosperity after a short period of activation (a bacterial concentration of about  $10^9$  -  $10^{12}$  CFU/ml). These results indicate a good sludge within the particles.

Taken together, the results of the NatiCap<sup>TM</sup> petroleum experiment showed an indirect reduction in PTH concentration (significant COD concentration reduction, up to 33 % reduction) in the aerobic stage of all of the treatment cycles. The most significant reduction within the COD values, occurred at the aerobic stage of cycle 3, indicating the effect of the microorganism age on their biodegradation capability. After 15 days of incubation, the microorganism's present a significant biodegradation rate. Significant increases in BOD and TSS (in both aerobic and anoxic stages) indicate a massive microorganism growth within the medium. Moreover, the NatiCap<sup>TM</sup> petroleum exhibited good biocompatibility after 1 week. The sludge exhibited within the particle was of good quality and the bacterial concentration within the particle was up to  $10^{12}$  cfu/ml. Furthermore, the biomass within the particles developed within a short period (3 days), which was efficient in treatment of petroleum wastewater, and the particle structure was stable and active for 3 weeks within the petroleum wastewater.

**EXAMPLE 5*****Municipal wastewater treatment – on site test 1*****MATERIALS AND EXPERIMENTAL PROCEDURES*****Product manufacture*****NatiCap<sup>TM</sup> petroleum**

Particles suitable for petroleum wastewater treatment were manufactured under semi-sterile conditions (designated herein as NatiCap<sup>TM</sup> petroleum) as follows:

1) The inner core components included: 2 glass beads, 2 dry inner cores (nutrient agar, LA), a commercial microbial blend (5 billion cfu/gram, USAbioproducts – Bactoclean PHC TYPE 4) which included selected adapted high potency microbes for biological biodegradation of petroleum hydrocarbons in, for example, petroleum refineries, chemicals, textile or pharmaceutical wastes, especially in activated sludge systems, and additional components including activated Carbon (dusk form). All of the inner core components were inserted into a water-soluble gelatin capsule (size: 000, Capsuline).

2) The gelatin capsule comprising all of the inner core components was coated with cellulose acetate 8 % polymer (15 ml per particle) as described in detail in Example 1, hereinabove.

**NatiCap<sup>TM</sup> municipal**

Particles suitable for municipal wastewater treatment, were manufactured under semi-sterile conditions (designated herein as NatiCap<sup>TM</sup> municipal) as follows:

1) The inner core components included: 2 glass beads, 2 dry inner cores (nutrient agar, LA), a commercial microbial blend (3 billion CFU/gram, USAbioproducts – Bactoclean FOG TYPE 2) which included a blend of dried microorganisms and enzymes designed to digest and decrease grease-comprising biomass in commercial and retail establishments such as grease traps, piping systems and lift stations, etc. All of the inner core components were inserted into a water-soluble gelatin capsule (size: 000, Capsuline).

2) The gelatin capsule comprising all of the inner core components was coated with cellulose acetate 8 % polymer (15 ml per particle) as described in detail in Example 1, hereinabove.

***Test model******Particle activation***

35 particles (30 capsules of NatiCap<sup>TM</sup><sub>Municipal</sub> and 5 capsules of NatiCap<sup>TM</sup><sub>Petroleum</sub>) were incubated for 48 hours in a test bioreactor. The particles were packaged in an elastic mesh bag and were immobilized to the center of the test bioreactor.

***System activating and stabilizing***

Two bioreactors were used (control and test bioreactors), as depicted in Figures 20 and 21B. Both bioreactors were filled with sludge and waste influents. As depicted in Figures 21A-B, the waste influents flowed continuously from a C110 pond (raw wastewater after anaerobic treatment) to the bioreactors (common feed - parallel to both bioreactors, 350 L) and at this stage the diffusers were activated. The hydraulic retention time (HRT) of the wastewater within the bioreactors was 5 days. The effluents (outlet) from the bioreactor flowed to a sedimentation tank (250 L) in the same flow rate of the influent (inlet) by overflow & gravitation. A diffuser and agitation were used for enrichment of the waste with oxygen (air flow pressure 0.5 Bar).

After stabilizing the systems, the 35 particles were added to the test system. Wastewater was introduced at rate flow of 70L/Day. Manual sludge refreshing was carried out every 3 days (1/4 of the sludge being returned to the bioreactor – RAS, the residual sludge was disposed out of the system - WAS). Samples for analysis were taken from the upper zone of the sedimentation tank (using a valve, see Figure 21B).

After 4 weeks of particle incubation, the particles were removed from the test system and were analyzed for shape, damage and for bacterial content (in their inner medium). The control and test bioreactors were monitored for another week. Thus, the total test period was about 5 weeks.

Analysis sampling for TOC (total organic compounds), BOD (biological oxygen demand), COD (chemical oxygen demand), oil and fat was carried out every 3-7 days. Chemical analysis was performed at the end of 5 weeks and the final analysis sample was also taken at the end of the 5 weeks.

After 5 weeks, at the end of the test (first cycle), both sedimentation tanks were emptied and cleaned. In order to homogenized the biomass within the bioreactors, 20 liters of bioreactor fluid was exchanged between the bioreactors every 3-5 days for two

weeks after terminating the first test cycle and prior to the second test cycle (Example 6, below).

## RESULTS

The on site test was carried out over a four weeks period in a wastewater treatment facility and included two bioreactor systems: the control system (representing the current treatment process comprising sludge and wastewater) and the test system (comprising the current treatment process of sludge and wastewater along with the 35 particles of the present invention).

The present inventor evaluated the quality improvement in the wastewater effluents (i.e. municipal wastewater).

### Chemical analysis results

The wastewater quality was monitored by obtaining samples from the sedimentation tanks using a valve (effluents) and from the anoxic pond (influent). The samples were transferred to the laboratories for further analysis. Table 6, below, summarizes the results of the chemical analysis and shows:

1. The organic load of the influents were high for typical municipal wastewater, this was probably due to the site plant drain of agriculture wastewater, slaughters, light industry (garage, metal processing industry, gas stations) and wastes of olive oil milling (during the period in which the test cycle was carried out).

2. On days 0-3 (i.e. up to 3 days after capsules activation) both the test and control bioreactors illustrated similarly good results of their influents. However, the effluents' COD and TOC (biodegradation measurements =  $1 - \text{effluents/influent} \times 100$ ), presented similar results in the test bioreactor compared to the control bioreactor (COD and TOC were 8-9 % and 3-8 % higher, respectively, in the particle comprising bioreactor) and the BOD analysis were significantly higher in the test bioreactor with a 16-22 % increase in BOD in the particle comprising bioreactor.

3. While the control bioreactor illustrated some form of water treatment, the particle comprising bioreactor (test system) illustrated better results in all of the parameters tested: average values of biodegradation (%) from the influents: COD - 85.5 (particle comprising bioreactor) vs. 68.8 (control bioreactor), BOD - 85.9 (particle comprising bioreactor) vs. 60.5 (control bioreactor), TOC - 91.7 (particle comprising bioreactor) vs. 73.8 (control bioreactor).

4. Importantly, the inventor of the present invention noted that the control bioreactor illustrated biological stress which occurred on day 6 of the trial and continued until day 24 of the trial (peak – days 10 to 17, Figure 22A). During the biological stress period, the control bioreactor's performance decreases (organic load reduction) up to: COD - 42.5 %, BOD - 34.3 %, TOC – 21.3% (1-Effluents/Influents x 100). During this time, the test bioreactor did not present any biological stress and its biodegradation capabilities were stable (Figure 22B).

5. The inventor noted that both bioreactors achieved an almost complete biodegradation (less than 10 mg/ml) of fat & oils, however, the concentration of these influents in the municipal wastewater treated was, in most cases, not high (13-152 mg/ml). Thus, since the values of oil & fats were not high, a complete analysis of this biodegradation using the test bioreactor could not be made. However, the TOC value presents total organic carbons which included oil, fats, detergents, solvents and more. The test bioreactor TOC results indicated that the particle comprising bioreactor was very efficient in treating these compounds (TOC's) within the wastewater.

**Table 6: Chemical analysis results of Influent & Effluents, pilot test 1**

| Control-Effluents         |     |     |     | Particles-Effluents |     |     |     | Influents from the Anaerobic pond |     |     |      | Days | Sample (month) |
|---------------------------|-----|-----|-----|---------------------|-----|-----|-----|-----------------------------------|-----|-----|------|------|----------------|
| OIL                       | TOC | BOD | COD | OIL                 | TOC | BOD | COD | OIL                               | TOC | BOD | COD  |      |                |
| less than 10              | 37  | 260 | 391 | less than 10        | 22  | 56  | 285 | 152                               | 528 | 910 | 1360 | 0    | Aug.           |
| less than 10              | 79  | 160 | 358 | less than 10        | 21  | 57  | 161 | 97                                | 714 | 646 | 2297 | 3    | Aug.           |
| less than 10              | 57  | 283 | 371 | less than 10        | 24  | 164 | 230 | 17                                | 411 | 740 | 984  | 6    | Sept.          |
| less than 10              | 92  | 327 | 461 | less than 10        | 28  | 105 | 202 | 42                                | 117 | 521 | 989  | 10   | Sept.          |
| less than 10              | 391 | 553 | 826 | less than 10        | 28  | 110 | 167 | 57                                | 573 | 842 | 1437 | 17   | Sept.          |
| less than 10              | 36  | 272 | 388 | less than 10        | 32  | 116 | 227 | 100                               | 158 | 875 | 1387 | 24   | Sept.          |
| less than 10              | 18  | 152 | 275 | less than 10        | 20  | 59  | 145 | 66                                | 796 | 917 | 2289 | 31   | Sept.          |
| <b>Capsules exclusion</b> |     |     |     |                     |     |     |     |                                   |     |     |      |      |                |
| 26                        | 35  | 297 | 362 | less than 10        | 13  | 140 | 155 | 13                                | 632 | 615 | 1546 | 38   | Oct.           |

*Sludge results*

Sludge sedimentation tests were performed in order to estimate the sedimentation characteristics of the mixture liquid (mixed liquor suspended solids, MLSS) of each bioreactor (SVI –sludge volume index test), by the ratio of sludge volume fraction to the liquid volume fraction, and to measure the velocity of sludge sedimentation in each of the bioreactors. As shown in Figures 23A-F, the sludge sedimentation volume (% of total volume) in both bioreactors was similar at the beginning of this test cycle. The total sludge was 25-30 ml (12.5 %-15 %) from 200 ml of bioreactors effluents.

Of note, after the sedimentation stage the water from the test bioreactor was clearer compared to the water from the control bioreactor, at the end of the test period. Furthermore, the sludge fraction of the test mixture liquid was higher in the control as compared to the test bioreactor (22.5 % - 10 %, Figures 23I, 23L). The presence of gray matter within the control sedimentation tank, can be used as an indicator for the higher suspended solids within the control bioreactor.

*Operational process: Foaming and overflow of the wastewater within the bioreactors*

During the test period the present inventor had noted a major difference between the test and control bioreactors in both:

1. Wastewater foaming and overflow – a major difference was evident in wastewater foaming and overflow during the test period and evaluation of the control & test bioreactors. Within the control bioreactor foam occurred and massive overflow was observed at several different time periods (at least 4 events), however, the test bioreactor demonstrated only one event of foaming and overflow. Figures 24A-B show the foam overflow marks on both the test and control bioreactors, respectively.

2. Sludge disposal from the sedimentation tanks – the test and control sedimentation tanks showed a major difference in the amount of sludge or raw sewage (gray matter) evident at the bottom of the tanks (excess sludge). The control bioreactor sedimentation was drained once a week and mainly contained raw sewage (gray matter). During each draining process, the present inventor had drained about 40 liters of raw sewage (gray matter). After draining the raw sewage the inventor had further disposed of 10 liters of sludge (brown matter). 4 liters of sludge were returned to the bioreactor.

However, in the test bioreactor comprising the particles, the inventor typically did not find raw sewage/gray matter (except for one occasion in which the present inventor drained 6 liters of raw sewage, gray matter). Furthermore, the present inventor had disposed of 10 liters of sludge (brown matter) from the test bioreactor of which 4 liters were returned to the bioreactor. Thus, taken together these results illustrate a major reduction in sludge in the particle comprising bioreactor. The differences in the gray matter within the test and control sedimentation tanks, indicated a better biodegradation process within the test bioreactor. The test bioreactor superiorly digests the organic matter (sewage) in comparison to the control bioreactor.

#### *Bacteriological results*

After 4 weeks of trial, the particles were pulled out from the test bioreactor and were characterized (Figures 25A-B).

1. All of the 35 particles remained normal and intact (i.e. unbroken). The semi-permeable outer membrane in all of the particles remained clear, stable and functional.

2. Liquids from the particles were removed and measured (total of 1.3-1.6 ml per particle) and illustrated high turbidity (indicated high microorganism concentration).

3. Viable counts were made in order to evaluate microorganism concentrations. In the NatiCap<sup>TM</sup><sub>Petroleum</sub> particles the microorganism concentration was  $5 \times 10^9$  CFU/ml. In the NatiCap<sup>TM</sup><sub>Municipal</sub> particles the microorganism concentration was  $5 \times 10^8$  CFU/ml. The particle bioreactor flora was  $3 \times 10^8$  CFU/ml. It is important to note, however, that the particle microorganism concentrations are probably much higher than those recorded as not all microorganisms can grow on LA nutrient medium. Taken together, these results indicate that in both types of particles high concentration of microorganisms were observed, suggesting that bacterial activity was high during the entire test period.

4. Gram-stains and a microscopic visualization of the bacteria indicated a significant divergence in the microorganism populations between the two different particles (i.e. municipal and petroleum particles) and in the natural flora of the bioreactor (data not shown). Taken together, these results indicate that the membrane separation of the particles (i.e. encapsulating the microorganism cultures) were effective and that microorganism trafficking across the particle membrane did not occur. Thus, insertion of

particles comprising microorganisms into the bioreactors increased of the microbial diversity within the bioreactor, which led to more productive biodegradation process.

### *Conclusions*

The on site test was conduct for over a month and included two bioreactors which differed only in the presence of the particles. During the first week, both bioreactors presented similar biodegradation capabilities for TOCs and CODs, however, biodegradation of BODs were evident from the beginning of the test period and were better in the particle comprising bioreactor.

In order to estimate the biomass within the bioreactors, the present inventor had preformed sedimentation tests of the waste within the bioreactors. Similar biomass was observed in both the control and test bioreactors at the middle of the test period. However, at the end of the test period, the present inventor had observed a double quantity of biomass within the control bioreactor as compared to the particle comprising bioreactor, suggesting that the use of particles can reduce the forming sludge volume. Thus, the increasing microbial diversity within the test bioreactor resulted in a better treatment capability of the biomass within the bioreactor and in a volume reduction in the solid phase of the sludge (gray matter).

Microbial analysis of the bioreactors and particles after the test period showed high bacterial concentrations within the particle capsules (up to  $5 \times 10^9$ ). Moreover, the bacterial concentration within the particles was higher in comparison to the bioreactor flora concentration. By using gram stains and observations with a light microscope, the present inventor concluded that the microbial culture compositions differed between the different particles and the bioreactor flora, suggesting an effective separation via the semi-permeable membrane. Moreover, since the microbial compositions in the particles differed from the bioreactors flora, it is highly considered that the particles contribute to the increased microbial diversity.

Thus, in conclusion, the test bioreactor comprising the particles of the present invention showed superior capability in stably treating wastewater. In this on site test, the wastewater treatment plant contained drainage as well as antimicrobial agents which most likely flowed from an olive press industry wastewater (which contained phenols and polyphenols). Since the test system (comprising the particles) kept its biodegradation capability stable while treating growth inhibitor substance wastewater,

these results indicate the resistance of the microorganisms within the particles (e.g. for anti microbial agents) as compared to the control bioreactor flora. The presence of two biological processes (sludge & particles) within the bioreactor increased the stability and resistance of the biological process.

## EXAMPLE 6

### *Municipal wastewater treatment –on site test 2*

#### MATERIALS AND EXPERIMENTAL PROCEDURES

As described in detail in Example 5, above.

#### RESULTS

The on site test was carried out over an eight week period in a wastewater treatment facility and included two bioreactor systems: the control system (representing the current treatment process comprising sludge and wastewater) and the test system (comprising the current treatment process of sludge and wastewater along with the 35 particles of the present invention).

The present inventor evaluated the quality improvement in the wastewater effluents (i.e. municipal wastewater).

#### Chemical analysis results

The wastewater quality was monitored by obtaining samples from the sedimentation tanks using a valve (effluents) and from the anoxic pond (influent). The samples were transfer to the laboratories for further analysis.

1. The organic load of the influents were high for typical municipal wastewater, this was probably due to the site plant drain of agriculture wastewater, slaughters, light industry (garage, metal processing industry, gas stations) and wastes of olive oil milling.

2. Both bioreactors (test & control) presented good biodegradation ability during the test period. However, the particle comprising bioreactor illustrated slightly better results in all of the parameters tested in comparison to the control bioreactor system (see Table 7, below): average values of biodegradation (%) from the influents: COD – 89 (particle comprising bioreactor) vs. 87 (control bioreactor), BOD - 89 (particle comprising bioreactor) vs. 85 (control bioreactor), TOC - 93 (particle comprising bioreactor) vs. 91 (control bioreactor).

3. The inventor noted that both bioreactors achieved an almost complete biodegradation (less than 10 mg/ml) of fat & oils. However, the test bioreactor presented slightly better results over the control system, mainly after 5 and 6 weeks from particles (capsule) activation. Since the concentration of oil & fats in the municipal wastewater treated was not high, a complete analysis of this biodegradation using the test bioreactor could not be made.

**Table 7: Chemical analysis results of Influent & Effluents, pilot test 2**

| Control-Effluents                                     |      |      |      | Particle-Effluents |      |      |      | Influents from the Anoxic pond |      |      |      |      |                |
|-------------------------------------------------------|------|------|------|--------------------|------|------|------|--------------------------------|------|------|------|------|----------------|
| OIL                                                   | TOC  | BO D | CO D | OIL                | TOC  | BO D | CO D | OIL                            | TOC  | BO D | CO D | Days | Sample (month) |
| <b>Prior Capsule introduction into the bioreactor</b> |      |      |      |                    |      |      |      |                                |      |      |      |      |                |
| 49                                                    | 60   | 256  | 467  | 11                 | 58   | 259  | 524  | 112                            | 211  | 1135 | 1457 | -15  | Oct.           |
| 30                                                    | 126  | 383  | 636  | 38                 | 105  | 239  | 534  | 23                             | 716  | 1517 | 2710 | -5   | Oct.           |
| 49                                                    | 65   | 310  | 541  | 41                 | 57   | 173  | 303  | 186                            | 565  | 605  | 2301 | 0    | Nov.           |
| <b>After Capsule activation</b>                       |      |      |      |                    |      |      |      |                                |      |      |      |      |                |
| 34                                                    | 66   | 145  | 334  | 20                 | 55   | 139  | 225  | 380                            | 117  | 521  | 989  | 3    | Nov.           |
| 47                                                    | 66   | 174  | 468  | 57                 | 49   | 130  | 303  | 123                            | 573  | 842  | 1437 | 10   | Nov.           |
| less than 10                                          | 47   | 88   | 443  | less than 10       | 55   | 63   | 400  | less than 10                   | 622  | 639  | 2785 | 17   | Nov.           |
| less than 10                                          | 85   | 40   | 285  | less than 10       | 76   | 39   | 270  | less than 10                   | 1164 | 544  | 4208 | 24   | Nov.           |
| less than 10                                          | 45.5 | 49   | 205  | less than 10       | 42.5 | 48   | 149  | less than 10                   | 754  | 730  | 2291 | 31   | Dec.           |
| 68                                                    | 14   | 31   | 110  | less than 10       | 20   | 73   | 168  | 192                            | 665  | 915  | 2620 | 40   | Dec.           |
| 28                                                    | 21   | 103  | 250  | less than 10       | 26   | 82   | 217  | 69                             | 765  | 901  | 1738 | 45   | Dec.           |
| less than 10                                          | 60   | 215  | 288  | less than 10       | 61   | 146  | 279  | 35                             | 557  | 1043 | 1788 | 53   | Dec.           |

*Sludge results*

Sludge disposal from the sedimentation tanks – the present inventor has noted a reduction in up to 75 % in the amount of sludge in the test bioreactor compared to the control bioreactor. The control sedimentation tank contained a significantly higher volume of gray matter, while within the test sedimentation tank, hardly any gray matter was identified within the sludge. These results are similar to the sludge results presented in example 5, above.

*Bacteriological results*

After 8 weeks of trial, the particles were pulled out from the test bioreactor and were characterized.

1. All of the 35 particles remained normal and intact (i.e. unbroken). The semi-permeable outer membrane in all of the particles remained stable and functional.

2. Liquids from the particles were removed and measured (total of 1.2-1.6 ml per particle) and illustrated high turbidity (indicated high microorganism concentration).

3. Viable counts were made in order to evaluate microorganism concentrations. In the NatiCap<sup>TM</sup><sub>Petroleum</sub> particles the microorganism concentration was  $4 \times 10^9$  CFU/ml. In the NatiCap<sup>TM</sup><sub>Municipal</sub> particles the microorganism concentration was  $8 \times 10^8$  CFU/ml. It is important to note, however, that the particle microorganism concentrations are probably much higher than those recorded as not all microorganisms can grow on LA nutrient medium. Taken together, these results indicate that in both types of particles high concentration of microorganisms were observed, suggesting that bacterial activity was high during the entire test period.

*Conclusions*

The on site test was conducted for over two months and included two bioreactors which differed only in the presence of the particles. During the test period both systems had improved water contamination, however, the test bioreactor comprising the particles of the present invention illustrated better chemical results.

In order to estimate the biomass within the bioreactors, the present inventor had performed sedimentation tests of the waste within the bioreactors. Similar biomass was observed in both bioreactors during the test period (by SVI tests of the bioreactor mixture liquid). However, major differences were observed in the sludge quantities within the sedimentation tanks during the entire duration of the trial. The sludge amount

(volume) within the test sedimentation tank comprising the particles was significant lower (1/5 of the sludge volume measured in the control sedimentation tank).

Microbial analysis of the bioreactors and particles after the test period showed high bacterial concentrations within the particle capsules (up to  $4 \times 10^9$ ). These high levels indicate that the microorganisms within the particles are still active after 2 months of activation.

Thus, in conclusion, the test bioreactor comprising the particles of the present invention showed superior capability in treating wastewater over a prolonged period of time.

## EXAMPLE 7

### *Whey wastewater treatment –on site test 3*

#### MATERIALS AND EXPERIMENTAL PROCEDURES

##### *Product manufacture*

##### *NatiCap<sup>TM</sup> municipal*

NatiCap<sup>TM</sup> municipal particles were manufactured as described in detail in Example 5, above.

##### *NatiCap<sup>TM</sup> food industry*

Particles suitable for treatment of food industry wastewater were manufactured under semi-sterile conditions (designated herein as NatiCap<sup>TM</sup> food industry) as follows:

1) The inner core components included: 2 glass beads, 2 dry inner cores (nutrient agar, LA), a commercial microbial blend (Kazanci Environmental Technics, DC0003) which included a blend bacteria designed to biodegrades high organic load wastewater, such as whey wastewater (dairies), oil mills, triglycerides and natural hydrocarbons. All of the inner core components were inserted into a water-soluble gelatin capsule (size: 000, Capsuline).

2) The gelatin capsule comprising all of the inner core components was coated with cellulose acetate 8 % polymer (15 ml per particle) as described in detail in Example 1, hereinabove.

***Test model***

As illustrated in Figures 26A-B, the test system included a single bioreactor (100 liter) comprising a ring diffuser and 35 particles (30 capsules of NatiCap<sup>TM</sup><sub>food industry</sub> and 5 capsules of NatiCap<sup>TM</sup><sub>municipal</sub>). According to this test system, the effluents from the bioreactor flowed to a sedimentation tank (i.e. clarifier - 100 L) - batch sequence model (once every 5-6 days, 100 liters of whey wastewater was added to the bioreactor) at the same flow rate of the influent (overflow & gravitation). The hydraulic retention time of the wastewater within the bioreactor was 5-6 days. A diffuser enriched the waste with oxygen by agitated the waste (air flow pressure 0.5 Bar).

***Particle activation***

35 particles (30 capsules of NatiCap<sup>TM</sup><sub>food industry</sub> and 5 capsules of NatiCap<sup>TM</sup><sub>municipal</sub>) were added to the bioreactor via a cartilage above the ring diffuser (as depicted in Figure 26C). The particles were incubated in the bioreactor containing the wastewater for 48 hours for activation thereof.

***System activating and stabilizing***

The particles were added to the bioreactor after the addition of the influents (i.e. whey waste – before the manufacture of Ricotta cheese) and the diffusers were activated prior to particle activation (as mentioned above). As the hydraulic retention time of the wastewater (effluents waste) was 5-6 days, the process was defined as a batch process and every 5-6 days 100 liters of influents were added, and 100 liters of effluents were drainage from the clarifier after sampling performance.

Activated sludge was allowed to growth within the bioreactor and half a liter of sludge was returned to the bioreactor from the clarifier between collection of the different batches. Of note, after a few weeks of testing, no sludge was evident within the sedimentation tank.

Analysis sampling was taken from the upper zone of the sedimentation tank (using a valve) at different time intervals. Thus, at the initiation of the trial sampling was obtained of the influents, after 6 days sampling was obtained from the clarifier (effluents) and influents and after 12 days sampling was obtained from the influents and effluents. Of note, the present inventor had noted a few instances of foaming overflow from the bioreactor (estimate water loss-30-40 Liters). This was solved by feeding of 1-2 a litter of whey, once every 3-4 days.

After 42 days of particle incubation within the bioreactor, the particles were removed from the test system and were analyzed (bacterial tests were performed on the inner medium of the capsules and intra-capsule viable counts and microscopy were carried out).

The total test period of was about 6 weeks.

## RESULTS

The on site test was carried out in a whey wastewater treatment facility and included a single bioreactor. The present inventor evaluated the quality improvement in the whey comprising wastewater effluents (i.e. food industry wastewater).

As evident in Figure 27A, the pH was slightly higher in the effluents compared to the influents, with values of 5 - 5.3 compared to 4.4 - 4.9, respectively. Moreover, the temperature recorded within the bioreactor was higher in comparison to the environment temperature (delta of about 4 – 5 °C, data not shown). Both, elevation of pH and temperature indicated bacterial activity. The average influents parameters were: COD 101,598 mg/L, BOD 52,409 mg/L and TOC 33,462 mg/L.

The COD was reduced in the effluents compared to the influents by an average of 40,000 mg/l in 7 days intervals (Figure 27B), the BOD was reduced in the effluents compared to the influents by an average of 20,000 mg/l in 7 days intervals (Figure 27C) and the TOC was reduced in the effluents compared to the influents by an average of 12,000 mg/l in 7 days intervals (Figure 27D).

### *Microbial analysis results*

After two months within the bioreactor, particles were withdrawn from the bioreactor and were analyzed. Moreover, water from the bioreactor was sampled in order to compare the microbial population of the bioreactor and that comprised within the particles.

The results of the analysis showed that after 8 weeks within the whey wastewater, the concentration of the viable bacterial populations within the particles were high (data not shown). Furthermore, the microbial population within the particles differed from that found in the bioreactor (data not shown), thus indicating an increase in microbial diversity within the bioreactor.

*Conclusion*

The present results demonstrated the ability of the particles of the present invention to treat highly polluted whey wastewater (COD values of about 100,000 mg/l and BOD values of about 50,000 mg/l). The whey wastewater was treated for about 7 days in a bioreactor chamber containing oxygen and 35 particles, followed by a 7 day period in a sedimentation chamber. This procedure reduced the COD of the whey wastewater by an average of 40,000 mg/l, the BOD by 20,000 mg/l and the TOC by 12,000 mg/l, in 7 days intervals. The present inventor contemplates that increasing the hydraulic retention time (HRT) of the system will bring better effluent quality. Furthermore, the present inventor contemplates that increasing the pH value of the influent (to above 6) will increase removal of organic waste, such as nitrogen compounds (due to nitrification and de-nitrification processes).

**EXAMPLE 8*****Pharmaceutical wastewater treatment*****MATERIALS AND EXPERIMENTAL PROCEDURES*****Product manufacture*****NatiCap<sup>TM</sup> petroleum**

NatiCap<sup>TM</sup> petroleum particles were manufactured as described in detail in Example 5, above.

**NatiCap<sup>TM</sup> pharmaceutical**

Particles suitable for treatment of pharmaceutical wastewater were manufactured under semi-sterile conditions (designated herein as NatiCap<sup>TM</sup> pharmaceutical) as follows:

- 1) The inner core components included: 2 glass beads and 2 on-site yeast blend culture units which were prepared as follows: yeast cultures were isolated from wastewater – a sample (100 µl) of the wastewater was spread on YPD agar plate (using a drigalski stick) and was incubated for 24-48 hours at 30 °C. The yeast colonies which grew on the YPD plates, were isolate and YPD cores were prepared as a nutrient carrier for the yeast blend cultures (the carriers contains the yeast culture mixture).

All of the inner core components were inserted into a water-soluble gelatin capsule (size: 000, Capsuline).

2) The gelatin capsule comprising all of the inner core components was coated with cellulose acetate 8 % polymer (15 ml per particle) as described in detail in Example 1, hereinabove.

### ***Test model***

The test model includes a test and a control system which differ in the presence of the particles. The test system includes a bioreactor (10 liter) comprising 10 particles (5 particles of NatiCap<sup>TM</sup><sub>pharmaceutical</sub> and 5 particles of NatiCap<sup>TM</sup><sub>petroleum</sub>).

## **EXAMPLE 9**

### ***Municipal wastewater full treatment study in a Membrane Biological Reactor (MBR) treatment plant***

## **MATERIALS AND EXPERIMENTAL PROCEDURES**

### ***Product manufacture***

#### ***NatiCap<sup>TM</sup> petroleum***

NatiCap<sup>TM</sup> petroleum particles were manufactured as described in detail in Example 5, above.

#### ***NatiCap<sup>TM</sup> municipal***

NatiCap<sup>TM</sup> municipal particles were manufactured as described in detail in Example 5, above.

### ***Municipal wastewater plant***

An on site test was carried out over a 9 months period in a Membrane Biological Reactor (MBR) wastewater treatment facility which had two identical bioreactors (designated herein pound A and pound B). In order to perform a controlled study, pound B was used as a test pound into which the particles were introduced while pound A served as the control. Pounds A and B shared a common inflow from an anoxic pound.

The wastewater treatment plant had two identical processes, namely, first the inflow was treated in a common anaerobic and anoxic pound and then it flowed into two identical bioreactors (830 cube meter each). In each bioreactor, after the digestive

process, the mix liquor was filtrated with microfiltration membranes (KOBOTA, Japan). Therefore, the quality of the effluents in this facility was considered high. Since a major portion of the organic matter was separated from the effluents using the microfiltration membrane, the hydraulic retention time of the wastewater within the bioreactors was considered very short, approximately 6 hours (2400 cube meter per day treated by each bioreactor).

### ***Particle introduction administration***

The particles were introduced by using perforated cartridges, 1 mm pores size mesh, contains up to 2000 particles in each (Figures 29A-C). The cartridges containing the particles were introduced near the bioreactor membranes, 1.5 meter below the water surface.

#### June- August:

1000 particles were introduced (500 NatiCap<sup>TM</sup><sub>Petroleum</sub> & 500 NatiCap<sup>TM</sup><sub>Municipal</sub>).

#### August – October:

3000 particles were introduced (1500 NatiCap<sup>TM</sup><sub>Petroleum</sub> & 1500 NatiCap<sup>TM</sup><sub>Municipal</sub>).

#### November - December:

4500 particles were introduced (1500 NatiCap<sup>TM</sup><sub>Municipal</sub> & 3000 NatiCap<sup>TM</sup><sub>Petroleum</sub>).

#### January –February:

The particles location and depth within the bioreactor were altered. Namely, the particles were introduced near the flow income (influent from the anoxic pond), up to 3 meters below the water surface.

6500 particles were introduced (3750 NatiCap<sup>TM</sup><sub>Municipal</sub> 2750 NatiCap<sup>TM</sup><sub>Petroleum</sub>).

### ***Systems activity monitoring***

In order to observe a similar start point of both bioreactors and in order to evaluate the performance of each bioreactor (pond A & pond B), both systems were monitored prior to the introduction of the particles (into pond B)

## RESULTS

In the first study, an estimation of the ratio of the number of particles to cube meter wastewater was carried out. The calibration test for the number of the requested particles ranged from about 1000 particles up to about 6500 particles. The particle exchange was carried out at a frequency of once every 2 to 3 months (depending on the microbiology analysis of the medium inside the particles).

**Table 8A: Chemical analysis results (mg/L)**

| Table 8A. Chemical analysis results (mg/L) of the Influent |          |      |     |     |     |     |
|------------------------------------------------------------|----------|------|-----|-----|-----|-----|
|                                                            | Influent |      |     |     |     |     |
| Capsule exchange                                           | Days     | COD  | BOD | TSS | OIL | TOC |
| 1000 capsules                                              | 0        |      |     |     |     |     |
|                                                            | 1        | 1260 |     |     |     |     |
|                                                            | 10       |      |     |     |     |     |
|                                                            | 15       | 1532 |     |     |     |     |
|                                                            | 17       | 1910 | 370 |     | 73  |     |
|                                                            | 22       | 2652 |     |     |     |     |
|                                                            | 24       | 1410 |     |     |     | 312 |
|                                                            | 29       | 2793 |     |     |     |     |
|                                                            | 31       | 3933 |     |     |     | 475 |
|                                                            | 36       | 1292 |     |     |     |     |
|                                                            | 38       | 6830 |     |     |     |     |
|                                                            | 43       | 1370 | 821 |     | 51  |     |
|                                                            | 45       | 1689 |     |     |     | 294 |
|                                                            | 50       | 2720 |     |     |     |     |
|                                                            | 52       |      |     |     |     |     |
| 3000 capsules                                              | 66       |      |     |     |     |     |
|                                                            | 68       |      |     |     |     |     |
|                                                            | 73       |      |     |     |     |     |
|                                                            | 75       |      |     |     |     |     |
|                                                            | 82       |      |     |     |     |     |
|                                                            | 87       | 1592 | 786 |     |     |     |
|                                                            | 94       | 1310 | 450 |     |     |     |
|                                                            | 101      | 1284 | 485 | 440 | 64  |     |
|                                                            | 104      |      |     | 520 |     |     |
|                                                            | 109      | 1680 | 495 | 500 |     |     |
|                                                            | 112      |      |     |     |     |     |
|                                                            | 116      | 1437 |     | 440 |     |     |

|                    |     |      |     |      |     |  |
|--------------------|-----|------|-----|------|-----|--|
|                    | 124 | 1713 | 413 | 590  | 132 |  |
|                    | 126 |      |     | 410  |     |  |
| 4500 capsules      | 131 | 1605 | 528 | 620  |     |  |
|                    | 139 | 1392 | 167 | 480  |     |  |
|                    | 145 | 1263 | 522 | 520  |     |  |
|                    | 148 |      |     | 560  |     |  |
|                    | 154 | 762  |     | 1160 |     |  |
|                    | 159 | 589  | 310 | 420  | 88  |  |
|                    | 166 | 1418 |     | 864  |     |  |
|                    | 168 | 1042 |     | 340  |     |  |
|                    | 173 | 1290 |     | 400  |     |  |
|                    | 175 |      |     | 380  |     |  |
|                    | 180 |      |     | 480  |     |  |
|                    | 182 | 1244 | 540 | 460  | 64  |  |
|                    | 185 | 1819 |     | 800  |     |  |
|                    | 190 |      |     | 340  |     |  |
|                    | 195 | 1349 |     | 520  |     |  |
|                    | 202 | 1392 |     | 500  |     |  |
|                    | 204 | 1195 |     | 400  |     |  |
|                    | 209 | 1485 |     | 660  |     |  |
|                    | 211 | 1074 |     | 480  |     |  |
| 6000 capsules      | 218 | 1538 | 710 | 500  |     |  |
|                    | 223 | 1198 |     | 380  | 117 |  |
|                    | 225 | 320  |     | 907  | 59  |  |
|                    | 230 | 1208 | 466 | 520  |     |  |
|                    | 232 | 1696 | 860 | 600  |     |  |
|                    | 234 | 1319 | 214 | 540  |     |  |
|                    | 235 | 1169 | 293 | 420  |     |  |
|                    | 240 | 1290 | 340 | 300  |     |  |
|                    | 247 | 1556 | 222 | 480  |     |  |
|                    | 249 | 1435 |     | 620  |     |  |
|                    | 254 | 1320 | 620 | 420  |     |  |
|                    | 261 | 474  |     |      |     |  |
|                    | 263 | 810  |     |      |     |  |
| Capsules excluding | 268 | 883  | 190 | 320  |     |  |
|                    | 270 | 1359 |     | 840  |     |  |

Table 8B. Chemical analysis results (mg/L) of Pound A and Pound B Effluents

| Capsule<br>exchange | Days | Pound B |     |     |     |       | Pound A |     |     |     |       |
|---------------------|------|---------|-----|-----|-----|-------|---------|-----|-----|-----|-------|
|                     |      | COD     | BOD | TSS | OIL | TOC   | COD     | BOD | TSS | OIL | MLSS  |
| 1000<br>capsules    | 0    | 26      | 0.8 | 10  | <5  | 17100 | 24      | 4.6 | 15  | <5  | 16800 |

|               |     |    |      |     |    |       |    |      |     |    |       |
|---------------|-----|----|------|-----|----|-------|----|------|-----|----|-------|
|               | 1   | 68 |      |     |    | 19700 | 29 |      | 5   |    | 18200 |
|               | 10  | 10 |      | 5   |    | 18200 | 23 |      | 5   |    | 16900 |
|               | 15  | 12 |      | 4   |    | 16200 | 24 |      | 4   |    | 16700 |
|               | 17  | 11 | 0.5  | 4   | <5 | 17000 | 10 | 0.9  | 4   | <5 | 17800 |
|               | 22  | 10 |      | 4   |    | 20000 | 22 |      | 4   |    | 18100 |
|               | 24  | 5  |      | 4   |    | 17500 | 5  |      | 4   |    | 20000 |
|               | 29  | 6  |      | 8   |    | 11000 | 10 |      | 4   |    | 12000 |
|               | 31  | 18 |      | 4   |    | 13000 | 10 |      | 4   |    | 11900 |
|               | 36  | 28 |      | 4   |    | 14000 | 28 |      | 4   |    | 14500 |
|               | 38  | 17 |      | 4   |    | 17500 | 17 |      | 4   |    | 15500 |
|               | 43  | 10 | 0.9  |     | <5 | 19000 | 10 | 1.3  |     | 6  | 21000 |
|               | 45  | 17 |      |     |    | 19300 | 27 |      |     |    | 18300 |
|               | 50  | 40 |      | 10  |    | 18300 | 23 |      | 10  |    | 8900  |
|               | 52  |    |      | 4   |    | 16600 |    |      | 4   |    | 15200 |
| 3000 capsules | 66  | 3  | 13   | 5   | 5  | 2200  | 8  | 20   | 5   | 5  | 2700  |
|               | 68  |    |      | 5   |    | 3750  |    |      | 5   |    | 3650  |
|               | 73  | 13 | <0.5 | 4   | 5  | 5300  | 4  | <0.5 | 4   | 5  | 5200  |
|               | 75  |    |      | 2.5 |    | 5500  |    |      | 2.5 |    | 5700  |
|               | 82  |    |      |     |    | 8700  |    |      |     |    | 8200  |
|               | 87  | 10 | <5   | 4   |    | 10700 | 12 | <5   | 4   |    | 9714  |
|               | 94  | 10 | <5   | 4   |    | 8800  | 10 | <5   | 4   |    | 8900  |
|               | 101 | 10 | 0.5  | 5   | 5  | 9200  | 10 | 0.5  | 5   | 5  | 9100  |
|               | 104 |    |      |     |    | 9600  |    |      |     |    | 10400 |
|               | 109 | 10 | 5    | 5   |    | 9000  | 10 | 5    | 5   |    | 10800 |
|               | 112 |    |      |     |    | 9300  |    |      |     |    | 11000 |
|               | 116 | 20 | 5    | 5   |    | 10400 | 28 | 5    | 5   |    | 11200 |
|               | 124 | 19 | 0.5  | 5   | 5  | 11400 | 19 | 0.5  | 5   | 5  | 11700 |
|               | 126 |    |      | 10  |    | 12500 |    |      | 15  |    | 10400 |
| 4500 capsules | 131 | 27 | 3.4  | 4   |    | 9300  | 27 | 20   | 8   |    | 9400  |
|               | 139 | 10 | 1.8  | 5   |    | 6900  | 10 | 1.9  | 5   |    | 7300  |
|               | 145 | 45 | 3.4  | 8   |    | 9600  | 47 | 1.5  | 4   |    | 9800  |
|               | 148 |    |      | 10  |    |       |    |      | 30  |    | 11300 |
|               | 154 | 61 |      | 4   |    | 13200 | 58 |      | 4   |    | 12800 |
|               | 159 | 25 | 5    | 5   | 5  | 11200 | 24 | 5    | 5   | 5  | 10600 |
|               | 166 | 70 | 72   | 6   |    | 13060 | 98 | 90   | 9   |    | 11580 |
|               | 168 | 59 |      | 4   |    | 12600 | 46 |      | 4   |    | 12200 |
|               | 173 | 16 |      | 5   |    | 13150 | 10 |      | 5   |    | 12050 |
|               | 175 |    |      | 5   |    | 13300 |    |      | 5   |    | 13900 |
|               | 180 |    |      | 5   |    | 18250 |    |      | 5   |    | 17800 |
|               | 182 | 70 | 9    | 5   | 10 | 18600 | 64 | 8    | 10  | 10 | 20300 |
|               | 185 | 30 |      | 5   |    | 27500 | 30 |      | 5   |    | 22600 |
|               | 190 |    |      | 4   |    | 24000 |    |      | 4   |    | 23600 |
|               | 195 | 47 |      | 5   |    | 29000 | 10 |      | 10  |    | 24800 |
|               | 202 | 10 |      | 4   |    | 23300 | 10 |      | 4   |    | 19700 |

|                    |     |    |   |    |    |       |    |    |    |    |       |
|--------------------|-----|----|---|----|----|-------|----|----|----|----|-------|
|                    | 204 | 30 |   | 5  |    | 9700  | 31 |    | 5  |    | 10200 |
|                    | 209 | 42 |   | 5  |    | 8500  | 31 |    | 5  |    | 8100  |
|                    | 211 | 10 | 5 | 5  | 10 | 11600 | 10 | 5  | 5  | 10 | 10800 |
| 6000 capsules      | 218 | 16 | 5 | 5  |    | 23300 | 10 | 5  | 10 |    | 19700 |
|                    | 223 | 10 | 5 | 10 | 10 | 8200  | 10 | 5  | 10 | 18 | 7100  |
|                    | 225 | 6  | 5 | 5  | 10 | 8900  | 8  | 5  | 5  | 10 | 9000  |
|                    | 230 |    |   |    |    |       |    | 9  | 5  |    |       |
|                    | 232 |    |   | 5  |    |       | 21 | 12 | 10 |    |       |
|                    | 234 | 56 |   | 10 |    |       | 10 |    | 10 |    |       |
|                    | 235 | 10 |   | 10 |    |       | 10 |    | 5  |    |       |
|                    | 240 | 10 |   | 5  |    |       | 10 | 5  | 5  |    |       |
|                    | 247 | 10 | 5 | 5  |    |       | 10 |    | 5  | 10 |       |
|                    | 249 | 10 |   | 10 |    |       | 10 |    | 15 |    |       |
|                    | 254 | 10 | 5 | 5  |    |       | 10 | 5  | 5  |    |       |
|                    | 261 |    |   |    |    |       |    |    |    |    |       |
|                    | 263 |    |   |    |    |       |    |    |    |    |       |
| Capsules excluding | 268 | 7  |   |    |    |       | 10 |    |    |    |       |
|                    | 270 | 10 |   | 5  |    |       | 10 |    | 10 |    |       |

Analysis sampling for BOD (biological oxygen demand), COD (chemical oxygen demand), MLSS (Mixture liquid suspended solids) and TSS (total suspended solids) was carried out. As illustrated in the results, the use of the particles did not significantly influence COD values (Table 8A-B, above, and Figure 28A). The BOD concentrations were reduced within the test bioreactor (pound B comprising the particles) in comparison to the control bioreactor (pound A) (Table 8, above, and Figure 28B). The highest BOD reduction was observed during MLSS concentration reduction (Table 8, above, and Figure 28B), in coloration to events which observed during day 66, and day 139 to the experiment. Thus, it was estimated that the use of 4500 particles presents an efficient (cost-effective) result. The differences in MLSS concentrations were not significant when comparing the test bioreactor and the control bioreactor (Table 8A-B, above, and Figure 28C). TSS concentrations were also significantly reduced in the test bioreactor (pound B after comprising 4500 particles) as compared to the control bioreactor (pound A) (Table 8A-B, above, and Figure 28D).

#### Microbial analysis results

After two to three months within the bioreactor, particles were withdrawn from the bioreactor and were analyzed. Moreover, water from the bioreactor was sampled in

order to compare the microbial population of the bioreactor and that comprised within the particles.

The results of the analysis showed that after up to 12 weeks within the municipal wastewater, the microbial population within the particles were differed from that found in the bioreactor (data not shown), thus indicating an increase in microbial diversity within the bioreactor, and an effective separation between the bacterial populations within the particle and the bioreactor medium bacterial populations.

Taken together, these results demonstrate that the use of the particles improves the bioprocess of municipal wastewater treatment, especially in the following parameters: BOD and TSS. The improvement in BOD concentration reduction was observed especially after a significant reduction of the MLSS concentration within the bioreactor. Moreover, the results demonstrated the optimization of using more than 4500 particles for the reduction of BOD and TSS concentrations in wastewater.

## EXAMPLE 10

### *Effluents quality improvement and total nitrogen reduction in purified wastewater*

#### MATERIALS AND EXPERIMENTAL PROCEDURES

##### *Product manufacture*

##### *NatiCap<sup>TM</sup> petroleum*

NatiCap<sup>TM</sup> petroleum particles were manufactured as described in detail in Example 5, above.

##### *NatiCap<sup>TM</sup> municipal*

NatiCap<sup>TM</sup> municipal particles were manufactured as described in detail in Example 5, above.

##### *NatiCap<sup>TM</sup> De-nitrification*

Particles suitable for de-nitrification\_were manufactured under semi-sterile conditions (designated herein as NatiCap<sup>TM</sup> De-nitrification) as follows:

- 1) The inner core components included: 2 glass beads, 2-4 dry inner cores (nutrient agar, LA), a commercial microbial blend for de-nitrification (nitrates to nitrogen gas) under anoxic conditions in municipal and industrial wastewater (USA

**Bioproducts**, type 7). All of the inner core components were inserted into a water-soluble gelatin capsule (size: 000, Capsuline).

2) The gelatin capsule comprising all of the inner core components was coated with cellulose acetate 8 % polymer (15 ml per particle) as described in detail in Example 1, hereinabove.

#### NatiCap<sup>TM</sup> Oxi-additive

Particles suitable for oxygen enrichment were manufactured under semi-sterile conditions (designated herein as NatiCap<sup>TM</sup> Oxi-additive) as follows:

1) The inner core components included only 5 gr of a solid oxygen release compound (ORC advanced® – oxygen release compound, REGENESIS), inserted into a water-soluble gelatin capsule (size: 000, Capsuline).

2) The gelatin capsule comprising all of the inner core components was coated with cellulose acetate 8 % polymer (15 ml per particle) as described in detail in Example 1, hereinabove.

### ***Test outline design and performance***

#### Test System

The bioreactor and the Sedimentation chamber were constructed from a 2 metric cube tank, which has 3 chambers, each 650 liters. The inflow arrived from a municipal wastewater treatment plant, after a purification process (e.g effluents), and flowed to the bioreactor (using a pump). The hydraulic retention time (HRT) of the water was 21.6 hours (1 L per minute) within the aerobic chambers and 10.8 hours within the anoxic chamber. Figures 30A-C present the test system that was used in the study.

#### Capsules introduction protocol

At time 0 (in May), 100 particles were introduced to the aerated bioreactor chamber (50 NatiCap<sup>TM</sup><sub>petroleum</sub> + 50 NatiCap<sup>TM</sup><sub>Municipal</sub>). The effluents from the bioreactor were overflowed to the second chamber into the sedimentation chamber. At the same time, additional 100 particles (NatiCap<sup>TM</sup><sub>De-nitrification</sub>) were introduced to the sedimentation chamber. Outflow volume metric was used to calibrate the income and outcome flow rate. No additional nutrients were added to the bioreactor or to the sedimentation chamber. The concentration of the Dissolved Oxygen (DO) within the bioreactor was calibrated up to 1.2 mg/L and within the sedimentation chamber, up to

0.5 mg/L (averaged 0.2 mg/L). The particles were entered into an active state after 2-4 days. About 2 months later, all the particles that were introduced at time 0 were removed, and 100 new NatiCap<sup>TM</sup><sub>Municipal</sub> particles and 2 NatiCap<sup>TM</sup><sub>Oxi-additive</sub> particles were placed in the first bioreactor chamber. In addition, 100 new NatiCap<sup>TM</sup><sub>De-nitrification</sub> were placed in the sedimentation tank (in addition to the old 100 NatiCap<sup>TM</sup><sub>De-nitrification</sub> particles).

About 2 months later, all particles were excluded from the test tank, investigated for their outer membrane condition (morphologic analysis) and microscopic observation was done to the inner medium in order to characterize the microbial population in several chosen particles.

After about 85 days from a test start point, some of the particles within the aerated pound were observed as damaged (from the vortex rotor). Therefore, 20 new particles (NatiCap<sup>TM</sup><sub>Petroleum</sub>) were introduced into the aerated pound (to exchange the damages particles). The damage of the particles had a significant influence on the test results.

#### ***Chemical analysis protocol***

Inflow and outflow were sampled and analyzed in a frequency of once a week, by Palgei-Maim. The following parameters were tested: COD, BOD, TOC, TSS, N<sub>total</sub>, NH<sub>4</sub>, NO<sub>3</sub>, NO<sub>2</sub>, Total Kjeldahl Nitrogen, P<sub>total</sub> (ppm), D.O and pH.

#### ***Microbial analysis protocol***

The microbial analysis test was performed in two aspects: the medium of the bioreactor and anoxic chamber, and the medium inside the particles. Those analyses were preformed in a frequency of once a month. The samples were analysis first in a light microscope; followed by a Gram stain procedure.

### **RESULTS**

The use of water within a cool tower (e.g. in an Electric Power Company) demands chlorination and bactericides use, in order to decrease microbial growth due to organic load which flows through the system. Cool towers evaporate water about 300 meter cubes per one hour and therefore the recycling process of the water is essential. The recycled water process aims to exclude the brines from the water. The raw water (effluents) for the cool towers coming from municipal waste water treatment plants after purification process, typically contains low concentration of organics (BOD), nitrogen

and ammonia (chemical analysis results are presented in table 9, below). However, the presence of ammonia and nitrogen within the raw water affects the recycling water process (reduces it from 10 times recycling to 6-8 times), while the need of increased chloride results in brine concentration elevation.

**Table 9: MWWTP effluents chemical analysis results (average values during the experiment period)**

| Average inflow parameters concentration (mg/l) $\pm$ STD<br>(Actual results of the test) | Analysis         |
|------------------------------------------------------------------------------------------|------------------|
| 8.01 $\pm$ 0.17                                                                          | pH               |
| 39.5 $\pm$ 32.3                                                                          | TSS              |
| 62.7 $\pm$ 32.5                                                                          | COD              |
| 9.7 $\pm$ 8.3                                                                            | BOD              |
| 32.2 $\pm$ 8.6                                                                           | TOC              |
| 42.0 $\pm$ 13.1                                                                          | N <sub>tot</sub> |
| 31.9 $\pm$ 8.9                                                                           | NH <sub>4</sub>  |
| 2.0 $\pm$ 1.8                                                                            | NO <sub>3</sub>  |
| 5.7 $\pm$ 1.2                                                                            | P <sub>tot</sub> |

#### Chemical analysis results

The experiment was conducted from May until October (4.5 months). The results of the test present a stable system performance, with a significant reduction in organic load and total Nitrogen concentration (presented in Figures 31A-C and 32A-B). The chemical analysis parameters can be summarized as follows:

**COD:** COD reduction of 6-95 % between the influent and effluent with an average of **46.7  $\pm$  26 % reduction**.

**BOD:** The influents contained a very low concentration of BOD (9.7 mg/L  $\pm$ 8.3 mg/L), which could not support a high concentration bacteria growth, and the metabolic conversion of nitrate to nitrogen gas.

**NH<sub>4</sub><sup>+</sup>:** A significant and a constant reduction in Ammonium (through a nitrification and microbial assimilation) was observed with a reduction of up to 54 % and an average of **37.9  $\pm$  17 % reduction**.

**NO<sub>3</sub>:** Nitrate concentration within the influent had various values between 0.01 and 11.4 mg/l. During the process the nitrate concentration increased up to 26 mg/l, indicating a significant nitrification process. The Nitrate within the effluent was almost

always higher in comparison to the influents, as a result of a successful nitrification process. Significant reduction in nitrate concentration was observed after day 40 (counted from the beginning of the test). This indicated a successful de-nitrification process. The de-nitrification process improved on day 40 of the test and up to the end of the test.

**TKN:** Total Kjeldahl Nitrogen indicates the sum of organic nitrogen, ammonia and ammonium. The results showed a significant reduction of total Kjeldahl Nitrogen with an average of  **$31.8 \pm 11.5$  % reduction** of the effluents in comparison to the influent.

**N total:** The total Nitrogen concentration of the effluent is the result of the nitrification and de-nitrification processes. The reduction in total Nitrogen was up to 33 mg/L, with a better reduction performance in the second half of the experiment in comparison to the first part. The average of total N concentration **reduction was  $22.3 \pm 9.2$  %** during the experiment.

Thus, the results of this study showed that the reduction in total Nitrogen was up to 33.3 % (Average of  $22.3 \pm 9.2$  %) with better reduction performance in the second part of the experiment (in comparison to the first part).

The reduction in Ammonia was up to 54 % with an average of  $37.9 \pm 17$  % and a similar performance in the first half and the second half of the experiment.

#### Microbial analysis results

The microbial analysis of the bioreactor and sedimentation chamber mediums and within the particles was conducted. The microbial test included viable counts of the medium inside the particles, and microscopic analysis, including life observation and a gram stain analysis.

Microbial examination had illustrated a bacterial population characterization difference between the inner particle mediums and outer bioreactor and sedimentation chamber mediums. This observation indicated a sufficient separation over time (2 months) of the introduced bacterial culture within the particles from the microorganisms within the bioreactor and sedimentation/anoxic chamber mediums. Moreover, during the particles' active state, there was a high bacterial concentration.

A method to decontaminate nitrogen compounds from water with a negligible organic matter was presented herein. The organic matter is necessary to provide a

source of energy in order to engage and allow the de-nitrification process. As the majority of the requested organic matter for the de-nitrification process was provided in the inner core of the particles, the need for external organic source is excluded and water secondary contamination is prevented.

**Table 10: Summary of chemical analysis values (mg/L) during the study**

| TSS-<br>Out | TSS<br>-In | CO<br>D-<br>Out | COD -<br>In | BOD-<br>Out | BOD-<br>In | NO3<br>-mid | Total<br>Nitrogen<br>(Out) | Total –<br>Nitroge<br>n (In) | NO3<br>-Out | NO3<br>-In | Ammonia<br>-Out | Ammonia<br>-in | day |
|-------------|------------|-----------------|-------------|-------------|------------|-------------|----------------------------|------------------------------|-------------|------------|-----------------|----------------|-----|
| 25          | 25         | 55              | 41          | 5           | 5          |             | 48                         | 47                           | 7.8         | 4.8        | 32.9            | 41.1           | 0   |
| 10          | 20         | 28              | 30          | 5           | 5          |             | 44                         | 43                           | 26          | 4.5        | 15.9            | 34.6           | 9   |
| 4           | 16         | 10              | 29          | 5           | 5          |             | 40                         | 48                           | 11.8        | 2          | 22.6            | 34.9           | 15  |
| 10          | 12         | 24              | 35          | 5           | 5          |             | 36                         | 34                           | 21.2        | 1.6        | 4.8             | 25.6           | 21  |
| 20          | 16         | 40              | 52          | 5           | 5          |             | 32                         | 34                           | 11.6        | 0.3        | 13.4            | 26.6           | 28  |
| 20          | 16         | 36              | 46          | 5           | 5          |             | 27                         | 32                           | 13.7        | 3.8        | 10.9            | 23             | 35  |
| 15          | 35         | 52              | 70          | 5           | 8          |             | 28                         | 30                           | 6.7         | 0.6        | 12.7            | 23.8           | 42  |
| 24          | 40         | 49              | 62          | 5           | 11         |             | 22                         | 32                           | 4.6         | 1          | 21.6            | 31.4           | 49  |
| 40          | 60         | 54              | 92          | 5           | 21         | 1.6         | 29                         | 43                           | 1.2         | 1          | 22.4            | 27.4           | 56  |
| 30          | 110        | 32              | 120         | 5           | 26         | 2.12        | 30                         | 39                           | 0.6         | 1.1        | 22.9            | 29.8           | 63  |
| 5           | 85         | 71              | 113         | 13          | 22         | 0.8         | 62                         | 77                           | 0.2         | 0.6        | 43              | 53             | 70  |
| 40          | 160        | 70              | 236         | 11          | 59         | 1.6         | 44.5                       | 57                           | 0.94        | 0.2        | 24.6            | 38.2           | 77  |
| 35          | 120        | 21              | 192         | 5           | 36         | 3.5         | 39                         | 45                           | 1           | 1          | 27.9            | 22.7           | 83  |
| 10          | 65         | 37              | 52          | 5           | 5          | 2.1         | 36                         | 34                           | 0.2         | 11.4       | 25.5            | 13.4           | 90  |
| 30          | 200        | 12              | 248         | 5           | 63         | 3           | 25                         | 37                           | 4.3         | 1          | 17.5            | 32.6           | 96  |
| 15          | 55         | 36              | 142         | 5           | 22         | 3.1         | 28                         | 42                           | 1.8         | 1          | 25.6            | 30.4           | 103 |
| 15          | 35         | 41              | 63          | 5           | 10         | 3.4         | 34                         | 49                           | 1.09        | 0.01       | 30.7            | 47.5           | 109 |
| 10          | 25         | 50              | 80          | 5           | 15         |             | 55                         | 72.6                         | 4.1         | 1          | 53.6            | 69             | 116 |
|             |            |                 |             |             |            |             | 45                         | 66.7                         | 3.7         | 1          | 31.2            | 61             | 123 |
|             |            | 36              | 97          | 5           | 20         |             | 56                         | 68                           |             |            | 33.3            | 63.2           | 130 |
|             |            | 134             | 95          |             |            |             | 67                         | 64*                          |             |            | 53              | 60*            | 137 |

Of note, the chemical analysis results (mg/L) of the study are presented. The particles were excluded from the aerated & anoxic chambers after 130 days of experiment. The chemical analysis results after 137 days indicate of insufficient reduction in ammonia, total nitrogen, and COD (increased).

\* Average value (the chemical analysis was not performed).

In- influents.

Out-effluents.

Mid- middle chamber.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

## WHAT IS CLAIMED IS:

1. A method of reducing nitrate overload in water, the method comprising contacting the water with a particle comprising
  - (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth;
  - (ii) an inner membrane being fabricated from a water-soluble polymer, said inner membrane surrounding said inner core and a population of dried microorganisms selected capable of facilitating de-nitrification of the water; and
  - (iii) an outer porous membrane surrounding said inner membrane, said outer porous membrane being insoluble in water,thereby reducing nitrate overload in water.
2. The method of claim 1 wherein the water is wastewater.
3. The method of claim 2, wherein said wastewater is selected from the group consisting of petroleum wastewater, municipal wastewater, pharmaceutical wastewater, nitrogen enriched wastewater and food industry wastewater.
4. The method of claim 1, wherein said contacting is effected under anaerobic conditions.
5. The method of claim 1, wherein said microorganisms are selected from the group consisting of *Alcaligenes*, *Pseudomonas*, *Methylobacterium*, *Bacillus*, *Paracoccus* and *Hyphomicrobium* and a combination of same.
6. The method of claim 1, wherein said contacting is effected in the absence of non-particle associated organic matter.
7. A method of purifying food industry wastewater, the method comprising contacting the wastewater with a particle comprising:

- (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth;
  - (ii) an inner membrane being fabricated from a water-soluble polymer, said inner membrane surrounding said inner core and a population of dried microorganisms; and
  - (iii) an outer porous membrane surrounding said inner membrane, said outer porous membrane being insoluble in water,
- thereby purifying the food industry wastewater.

8. A method of purifying pharmaceutical wastewater, the method comprising contacting the wastewater with a particle comprising:

- (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth;
  - (ii) an inner membrane being fabricated from a water-soluble polymer, said inner membrane surrounding said inner core and a population of dried microorganisms; and
  - (iii) an outer porous membrane surrounding said inner membrane, said outer porous membrane being insoluble in water,
- thereby purifying the pharmaceutical wastewater.

9. A method of purifying municipal wastewater, the method comprising contacting the municipal wastewater with a plurality of particles, wherein said plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater, under conditions that allow said microorganisms to decontaminate the municipal wastewater, thereby purifying the municipal wastewater.

10. A method of purifying food industry wastewater, the method comprising contacting the food industry wastewater with a plurality of particles, wherein said plurality of particles comprise a first population of dried microorganisms selected for purification of food industry wastewater and a second population of dried microorganisms selected for purification of municipal wastewater, under conditions that

allow said microorganisms to decontaminate the food industry wastewater, thereby purifying the food industry wastewater.

11. A method of purifying pharmaceutical wastewater, the method comprising contacting the pharmaceutical wastewater with a plurality of particles, wherein said plurality of particles comprise a first population of dried microorganisms selected for purification of pharmaceutical wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater, under conditions that allow said microorganisms to decontaminate the pharmaceutical wastewater, thereby purifying the pharmaceutical wastewater.

12. A method of treating water, the method comprising contacting the water with a plurality of particles, wherein said plurality of particles comprise:

(i) a first population of dried microorganisms selected for purification of municipal wastewater;

(ii) a second population of dried microorganisms selected for purification of petroleum wastewater; and

(iii) a third population of dried microorganisms selected for de-nitrification of water;

under conditions that allow said microorganisms to purify the water, thereby treating the water.

13. The method of claim 12, wherein contacting with (i) and (ii) are effected simultaneously or sequentially.

14. The method of claim 12, wherein contacting with (i) and (ii) are effected under aerobic conditions.

15. The method of claim 12, wherein contacting with (iii) is effected under anaerobic conditions.

16. The method of claim 12, wherein contacting with (iii) is effected following (i) and (ii).

17. The method of claim 12, further comprising at least one particle suitable for oxygen enrichment.

18. The method of claim 17, wherein contacting with (i) and (ii) and said particles suitable for oxygen enrichment are effected simultaneously.

19. The method of claim 14, wherein said aerobic conditions comprise the presence of at least one particle suitable for oxygen enrichment.

20. The method of claim 12, wherein the water is wastewater.

21. The method of claim 20, wherein said wastewater is nitrogen enriched wastewater.

22. An article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater.

23. An article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of food industry wastewater and a second population of dried microorganisms selected for purification of municipal wastewater.

24. An article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of pharmaceutical wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater.

25. An article of manufacture comprising a plurality of particles, wherein the plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater, a second population of dried microorganisms selected for purification of petroleum wastewater and a third population of dried microorganisms selected for de-nitrification of wastewater.

26. The article of manufacture of claim 25, further comprising at least one particle suitable for oxygen enrichment.

27. An article of manufacture comprising a plurality of particles, wherein the plurality of particles comprises a population of dried microorganisms selected for de-nitrification of wastewater.

28. A method of reducing sludge production during wastewater purification, the method comprising contacting the wastewater with a plurality of particles, wherein said plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater, under conditions that allow said microorganisms to decontaminate the wastewater, thereby reducing sludge production during the wastewater purification.

29. A method of stabilizing a wastewater purification treatment, the method comprising contacting the wastewater with a plurality of particles, wherein said plurality of particles comprise a first population of dried microorganisms selected for purification of municipal wastewater and a second population of dried microorganisms selected for purification of petroleum wastewater, under conditions that allow said microorganisms to decontaminate the wastewater, thereby stabilizing the wastewater purification treatment.

30. The method of claims 17, 28 or 29, wherein said wastewater comprises municipal wastewater.

31. The method of claims 17, 28 or 29, wherein said wastewater is selected from the group consisting of petroleum wastewater, municipal wastewater, pharmaceutical wastewater, nitrogen enriched wastewater and food industry wastewater.

32. The method of any of claims 9-11, 28 or 29, or article of manufacture of any of claims 22-24, wherein said plurality of particles comprise at least two non-identical particles.

33. The method of any of claims 9-11, 28 or 29, or article of manufacture of any of claims 22-24, wherein a ratio of said first population of dried microorganisms and said second population of dried microorganisms is selected from the group consisting of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 and 10:1.

34. The method of any of claims 9-10, 28 or 29, or article of manufacture of any of claims 22-23, wherein a ratio of said first population of dried microorganisms and said second population of dried microorganisms is 6:1.

35. The method of claim 11, or article of manufacture of claim 24, wherein a ratio of said first population of dried microorganisms and said second population of dried microorganisms is 1:1.

36. The method of claim 12 or article of manufacture of claim 25, wherein a ratio of said first population of dried microorganisms, said second population of dried microorganisms and said third population of dried microorganisms is selected from the group consisting of 1:1:1, 1:1:2, 1:1:3, 1:1:4, 1:1:5, 1:1:6, 1:1:7, 1:1:8, 1:1:9 and 1:1:10.

37. The method of claim 12 or article of manufacture of claim 25, wherein a ratio of said first population of dried microorganisms, said second population of dried microorganisms and said third population of dried microorganisms is 1:1:2.

38. The method of any of claims 9-12, 28 or 29, wherein said plurality of particles is selected such that the ratio of said particles to said wastewater is between about 0.01 to 5 particles per cube wastewater per day.

39. The method of any of claims 9-12, 28 or 29, or article of manufacture of any of claims 22-26, wherein each of said particles comprise:

- (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth;
- (ii) an inner membrane being fabricated from a water-soluble polymer, said inner membrane surrounding said inner core and said dried microorganisms; and
- (iii) an outer porous membrane surrounding said inner membrane, said outer porous membrane being insoluble in water.

40. The method of any of claims 9-12, 28 or 29, wherein said particles are pre-activated in a liquid prior to said contacting.

41. The method of claim 40, wherein said particles are pre-activated for about 24 to 96 hours prior to said contacting.

42. The method of any of claims 9-12, 28 or 29, wherein said contacting is effected for a period of about 2 weeks to about 45 weeks.

43. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said solid matrix of nutrients comprises an agar.

44. The method of any of claims 1-12, 28 or 29, or article of manufacture of any of claims 22-26, wherein said microorganisms comprise bacteria.

45. The method or article of manufacture of claim 44, wherein said bacteria comprise freeze-dried bacteria.

46. The method of any of claims 1-12, 28 or 29, or article of manufacture of any of claims 22-26, wherein said microorganisms comprise yeast.

47. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said inner core is coated with a control release polymer.

48. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said inner membrane or said inner core further comprise an enzyme.

49. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said inner core further comprises an agent selected from the group consisting of an amino acid, an enzyme, a peptide, a protein, a carbohydrate, a sugar, an iron, a salt and an essential element.

50. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said inner core is devoid of at least one agent selected from the group consisting of an amino acid, a peptide, a protein, a carbohydrate, a sugar, an iron, a salt and an essential element.

51. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said particles support biofilm formation within.

52. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said inner membrane further comprises additional elements which support biofilm formation thereon.

53. The method or article of manufacture of claim 52, wherein said additional elements comprise glass beads.

54. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said inner membrane further comprises activated carbon granules or activated carbon chips within.

55. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said inner membrane further comprises an oxygen release compound within.

56. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said water-soluble polymer comprises gelatin.

57. The method or article of manufacture of claim 56, wherein said gelatin is further coated with a control release polymer.

58. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said outer porous membrane is fabricated from a polymer selected from the group consisting of PVAL (polyvinyl-alcohol), Polyethersulfone (PES), Cellulose Acetate, Cellulose Nitrate, Ethyl Cellulose, Nitrocellulose Mixed Esters, Polycarbonate film, Nylon, PVDF(poly(vinylidene fluoride)) and Polysulfone.

59. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said outer porous membrane is fabricated from a polymer comprising Cellulose Acetate.

60. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein said porous membrane is resistant to biofilm formation.

61. The method or article of manufacture of any of claims 1, 7, 8 or 39, wherein a pore of said porous membrane is less than 0.85  $\mu\text{M}$ .

62. The method of any of claims 1-12, 28 or 29, or article of manufacture of any of claims 22-26, wherein said particle is between 0.5-30 cm in length.

63. A particle suitable for de-nitrification, comprising:

- (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth;

- (ii) an inner membrane being fabricated from a water-soluble polymer, said inner membrane surrounding said inner core and a population of dried microorganisms, wherein said dried microorganisms are selected for facilitating de-nitrification; and
- (iii) an outer porous membrane surrounding said inner membrane, said outer porous membrane being insoluble in water.

64. A particle suitable for purification of municipal wastewater, comprising:

- (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth;
- (ii) an inner membrane being fabricated from a water-soluble polymer, said inner membrane surrounding said inner core and a population of dried microorganisms, wherein said dried microorganisms are selected for purification of municipal wastewater; and
- (iii) an outer porous membrane surrounding said inner membrane, said outer porous membrane being insoluble in water.

65. A particle suitable for purification of food industry wastewater, comprising:

- (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth;
- (ii) an inner membrane being fabricated from a water-soluble polymer, said inner membrane surrounding said inner core and a population of dried microorganisms, wherein said dried microorganisms are selected for purification of food industry wastewater; and
- (iii) an outer porous membrane surrounding said inner membrane, said outer porous membrane being insoluble in water.

66. A particle suitable for purification of pharmaceutical wastewater, comprising:

- (i) at least one inner core which comprises a solid matrix of nutrients for microorganism growth;

- (ii) an inner membrane being fabricated from a water-soluble polymer, said inner membrane surrounding said inner core and a population of dried microorganisms, wherein said dried microorganisms are selected for purification of pharmaceutical wastewater; and
- (iii) an outer porous membrane surrounding said inner membrane, said outer porous membrane being insoluble in water.

67. A particle suitable for oxygen enrichment, comprising:

- (i) at least one inner core which comprises a solid oxygen release compound;
- (ii) an inner membrane being fabricated from a water-soluble polymer, said inner membrane surrounding said inner core; and
- (iii) an outer porous membrane surrounding said inner membrane, said outer porous membrane being insoluble in water.

68. The particle of claim 67, being devoid of microorganisms.

69. The particle of claim 67, comprising microorganisms surrounded by said inner membrane.

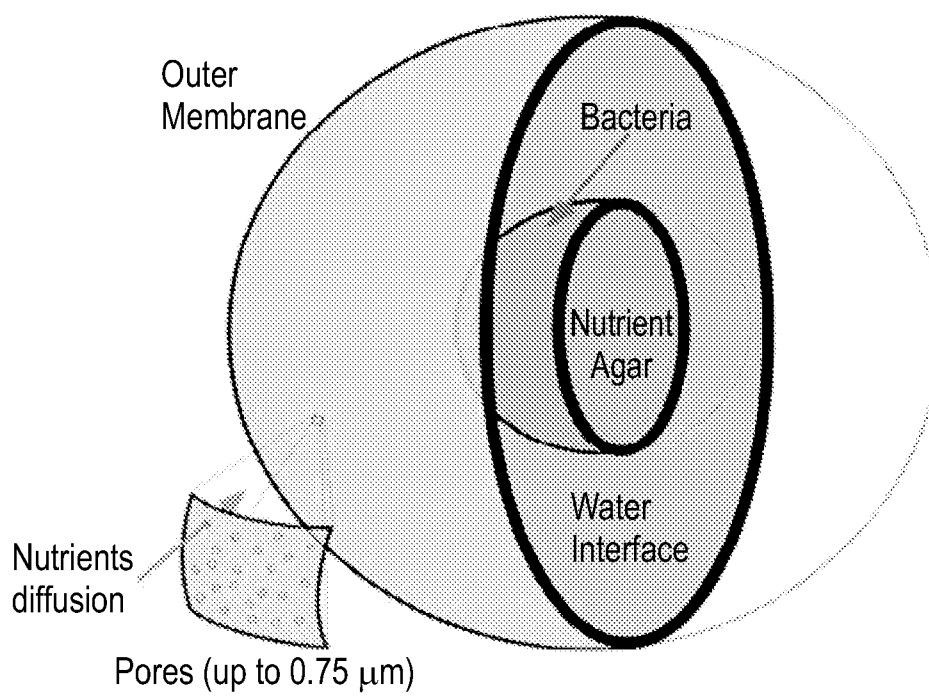


FIG. 1A

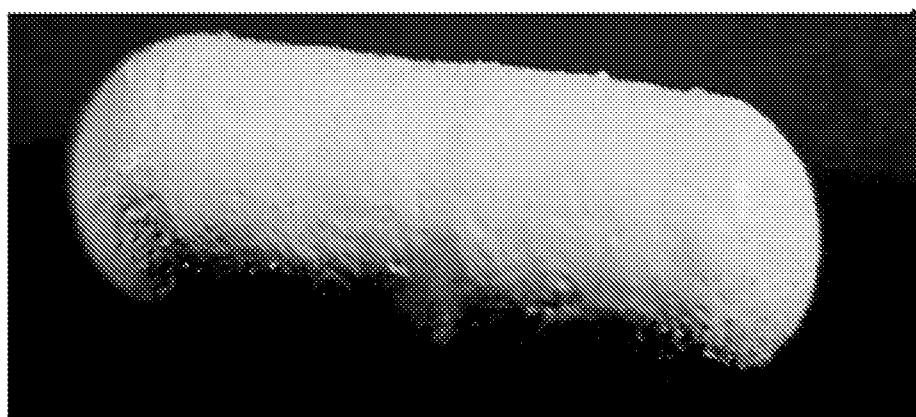


FIG. 1B

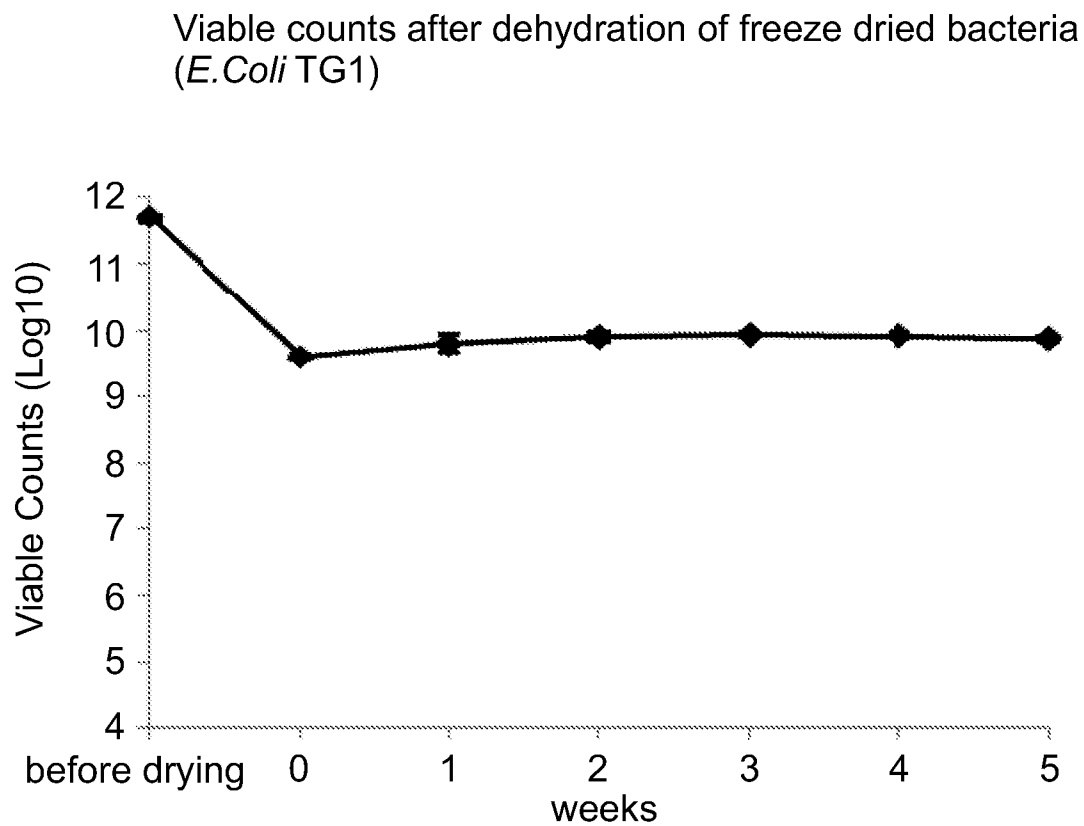


FIG. 2

FIG. 3



FIG. 4B

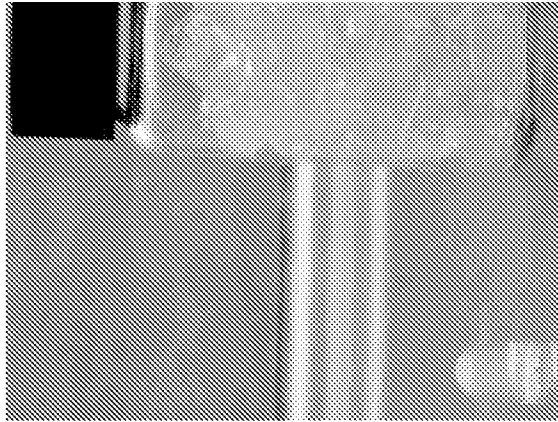


FIG. 4A

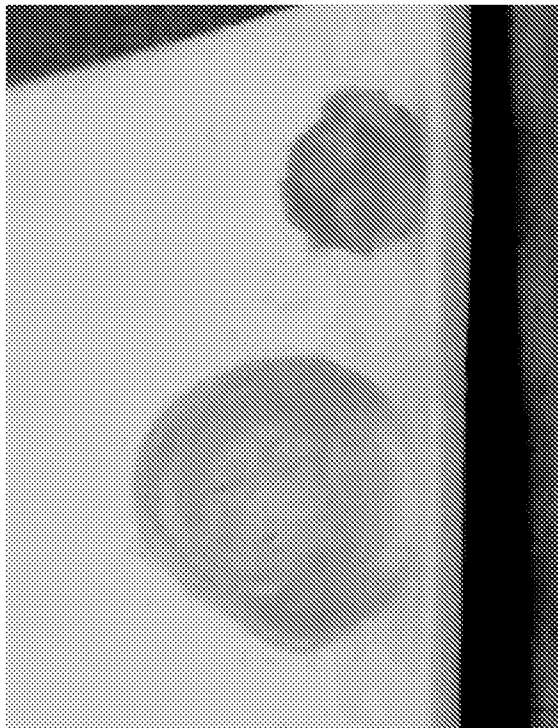
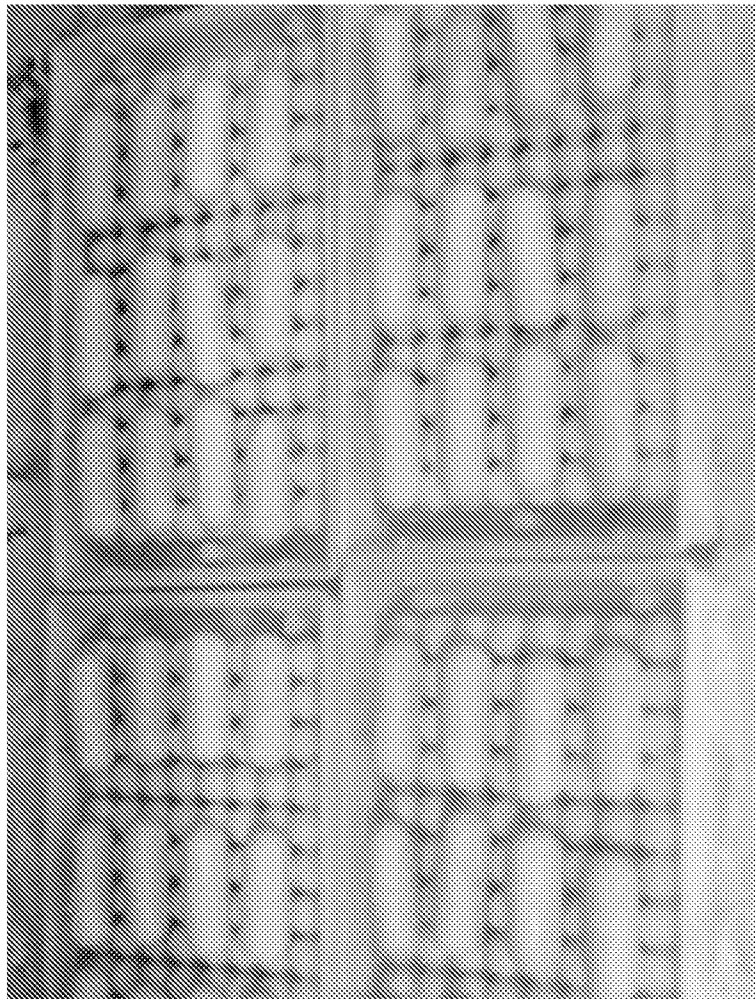


FIG. 5



FIG. 6



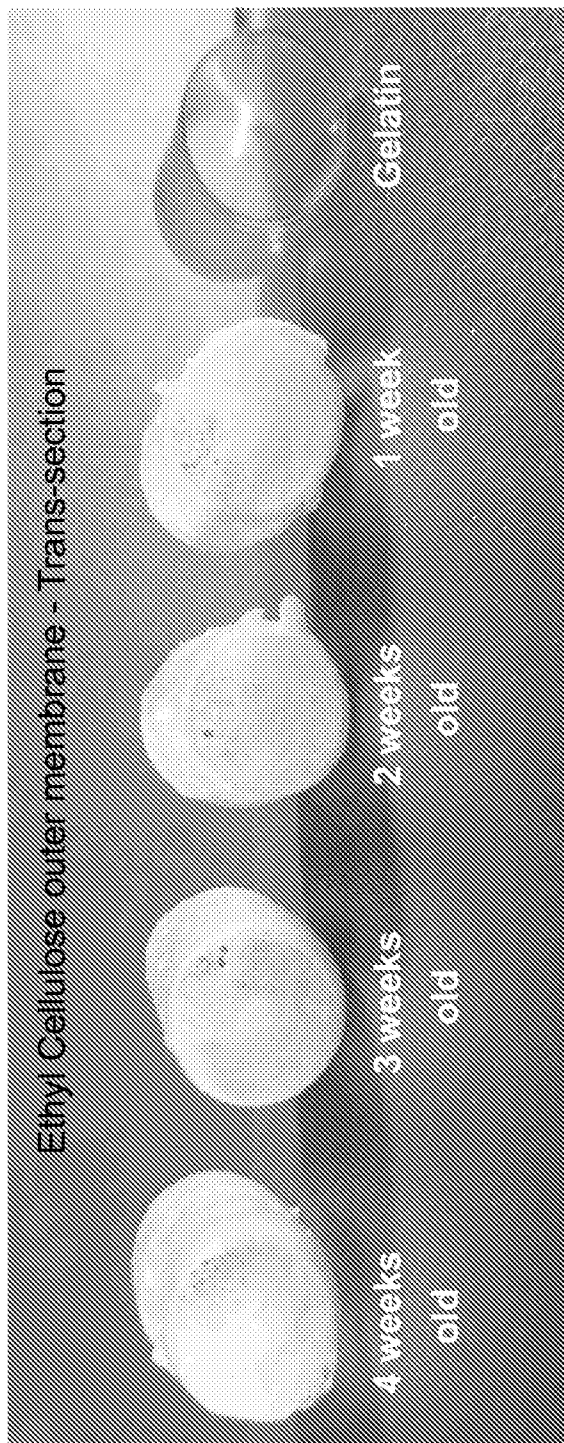


FIG. 7A

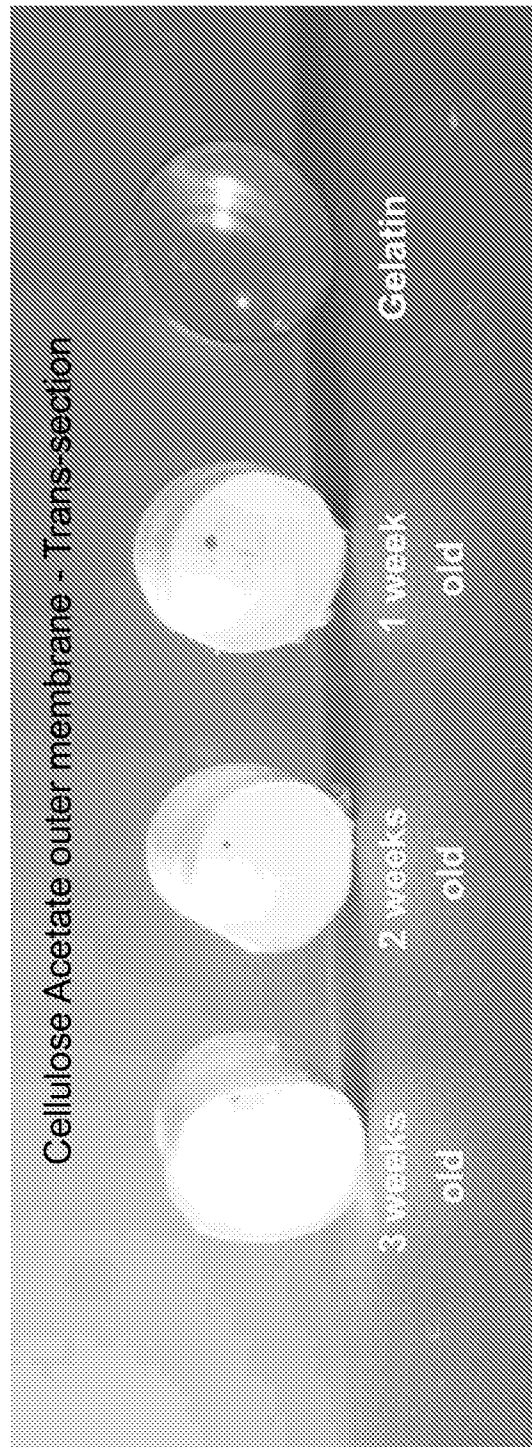


FIG. 7B

Water flow rate into Ethyl Cellulose (8%) coated capsules

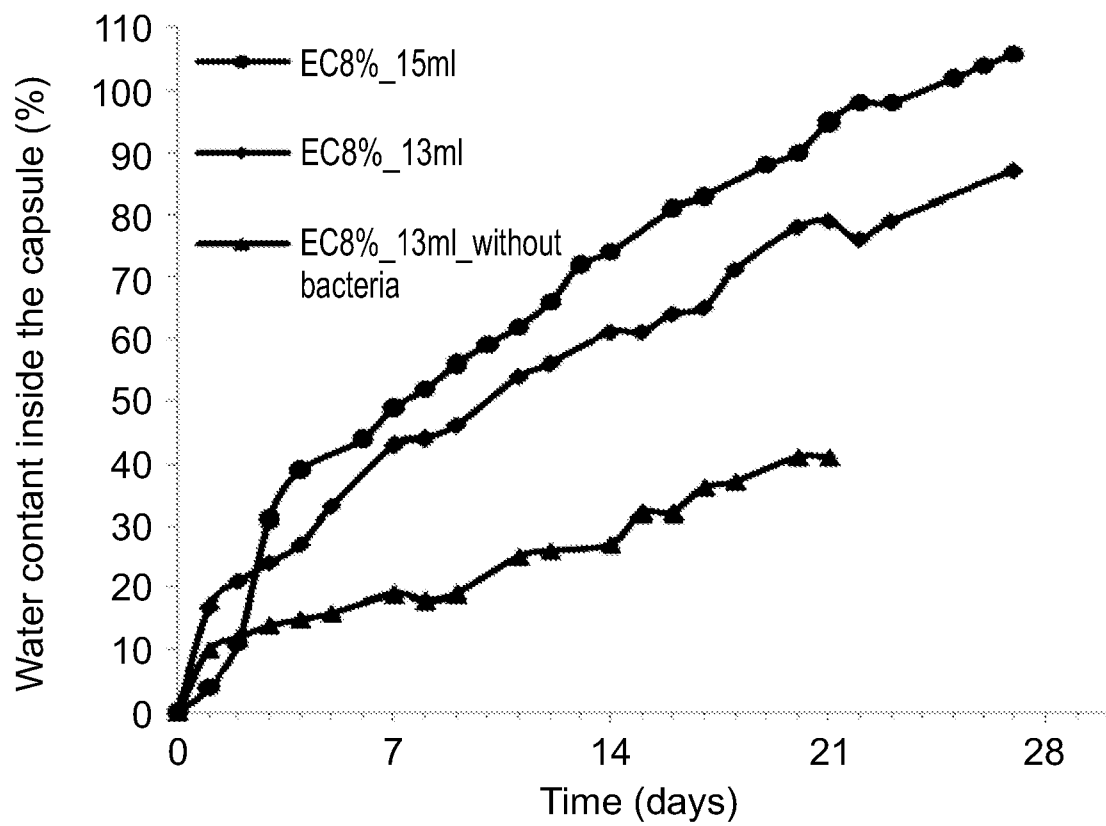


FIG. 8

Water flow rate into a Cellulose Acetate (8%) coated capsules

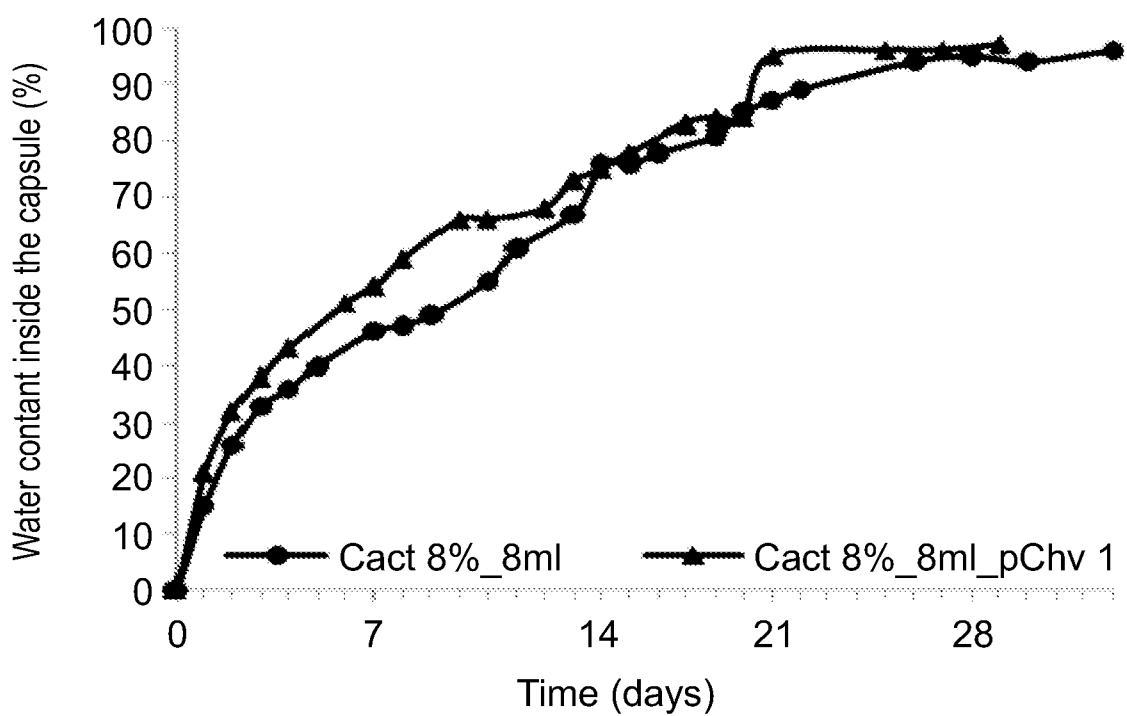


FIG. 9

Comparison of water flow rate into Ethyl Cellulose and Cellulose Acetate coated capsules

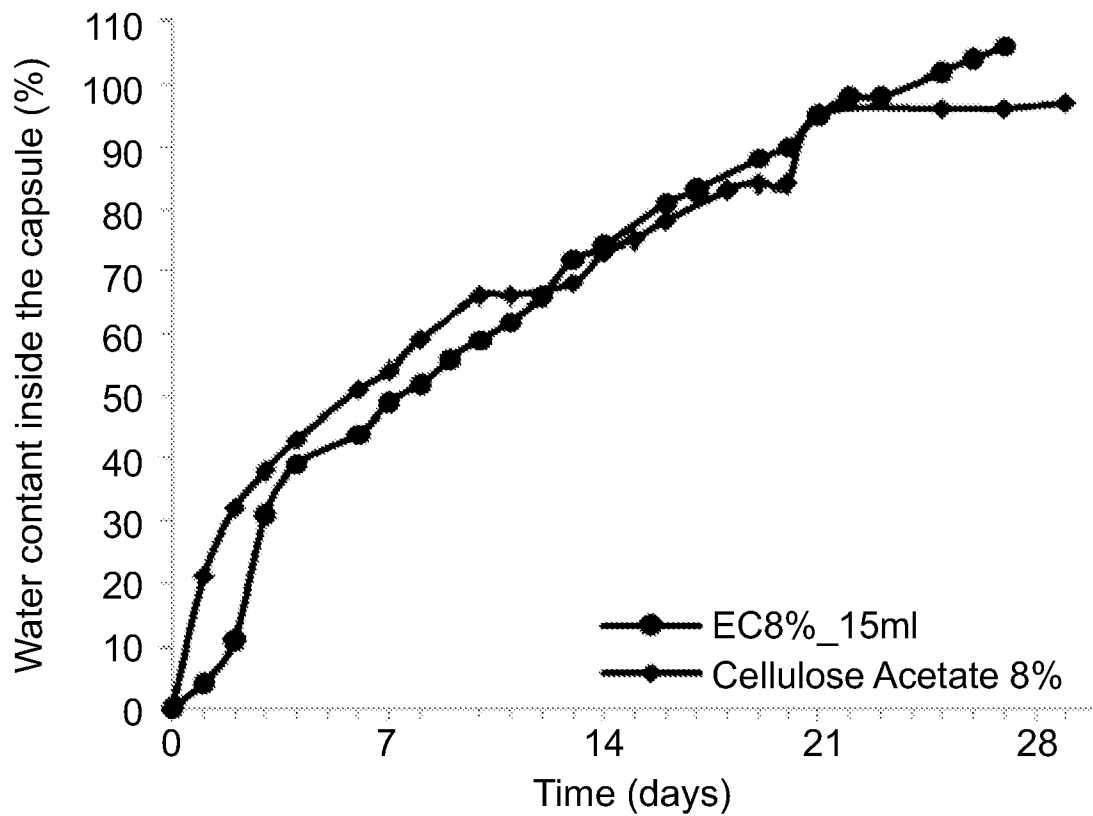


FIG. 10

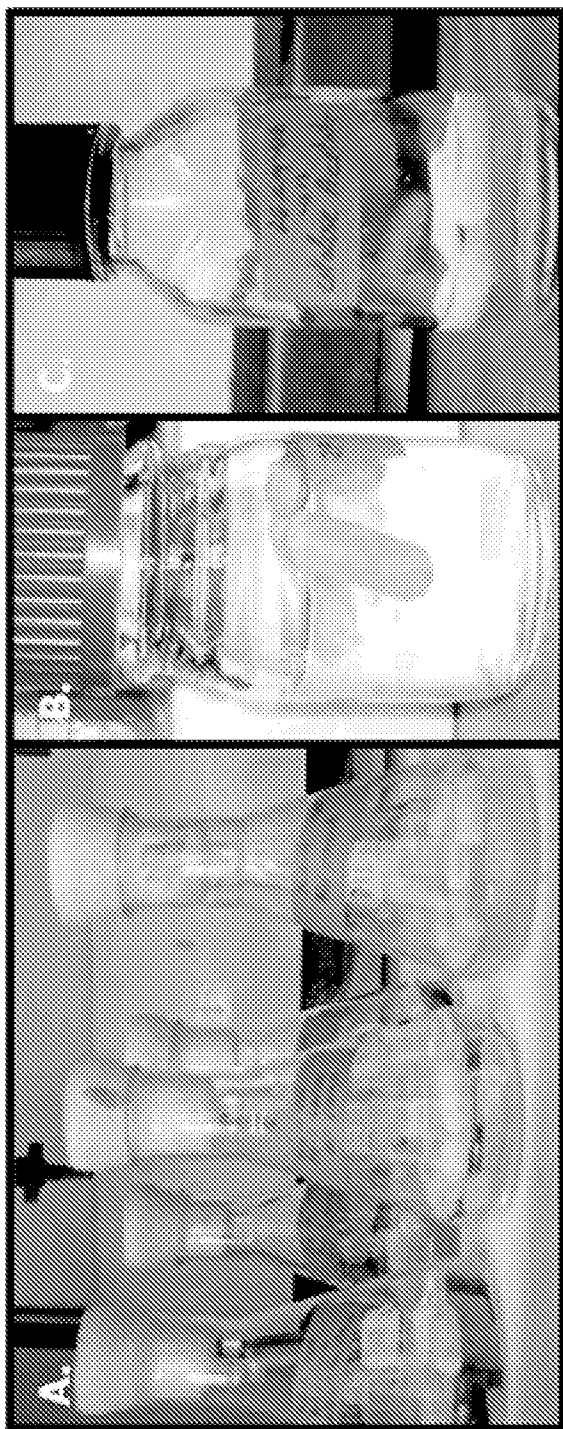


FIG. 11A      FIG. 11B      FIG. 11C

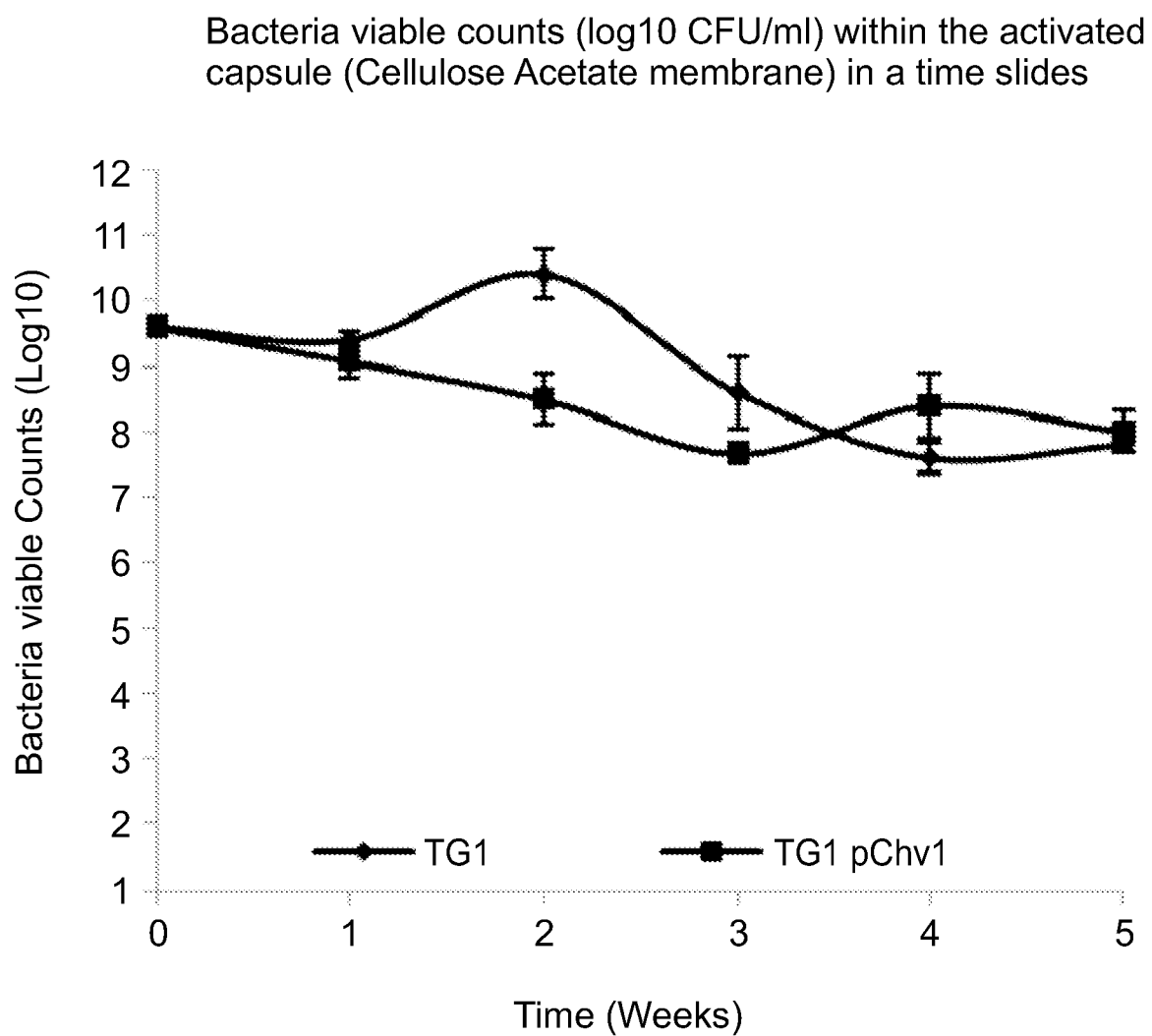


FIG. 12

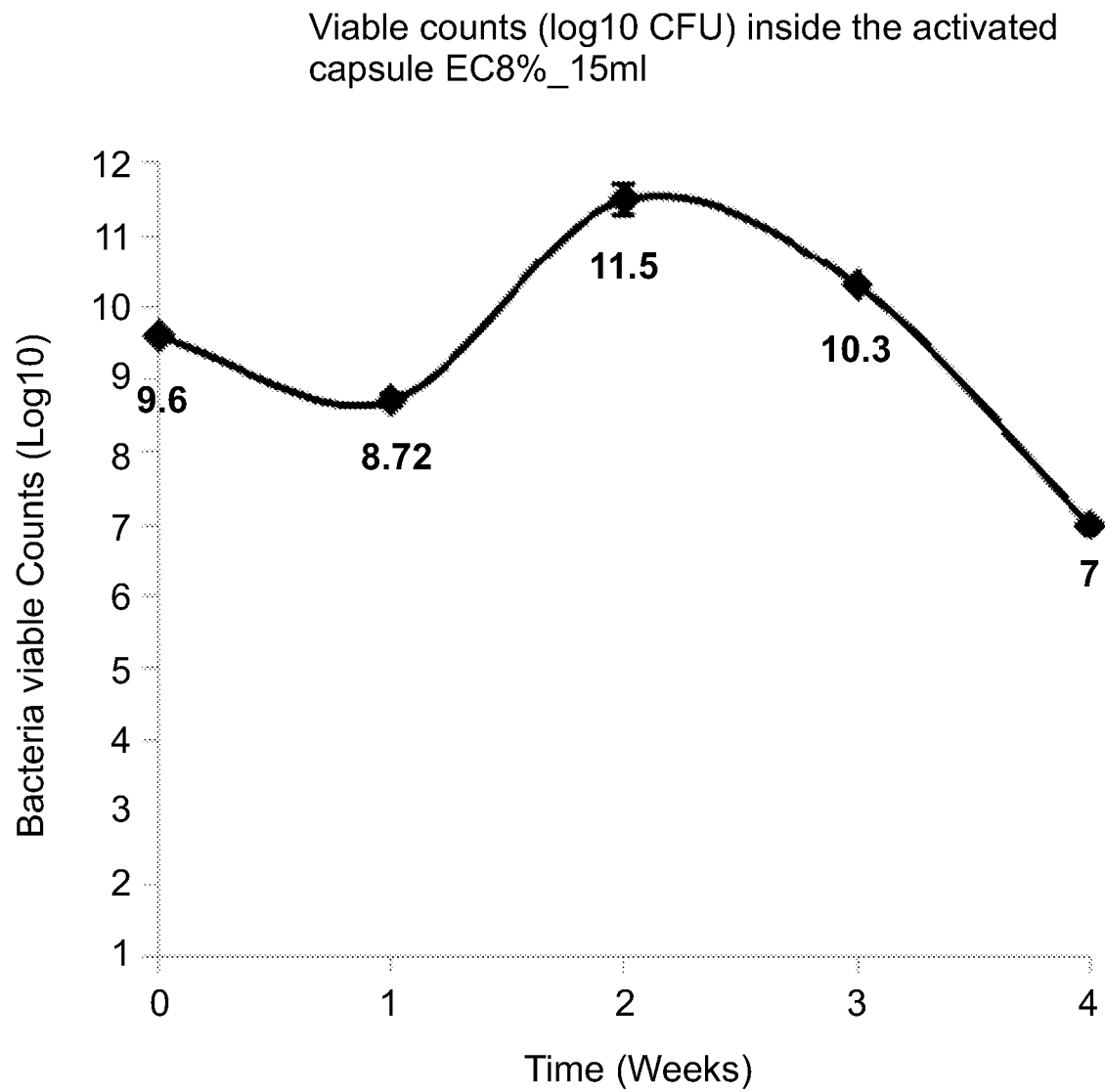
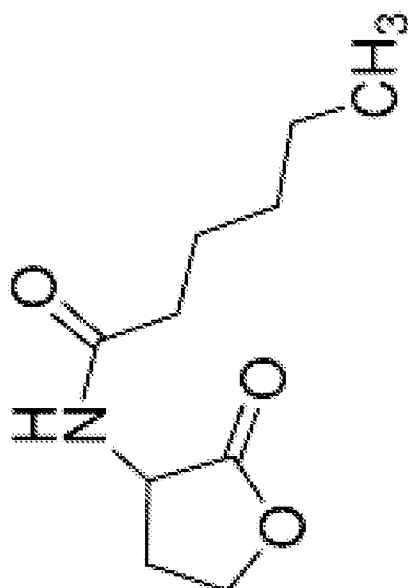


FIG. 13

FIG. 14



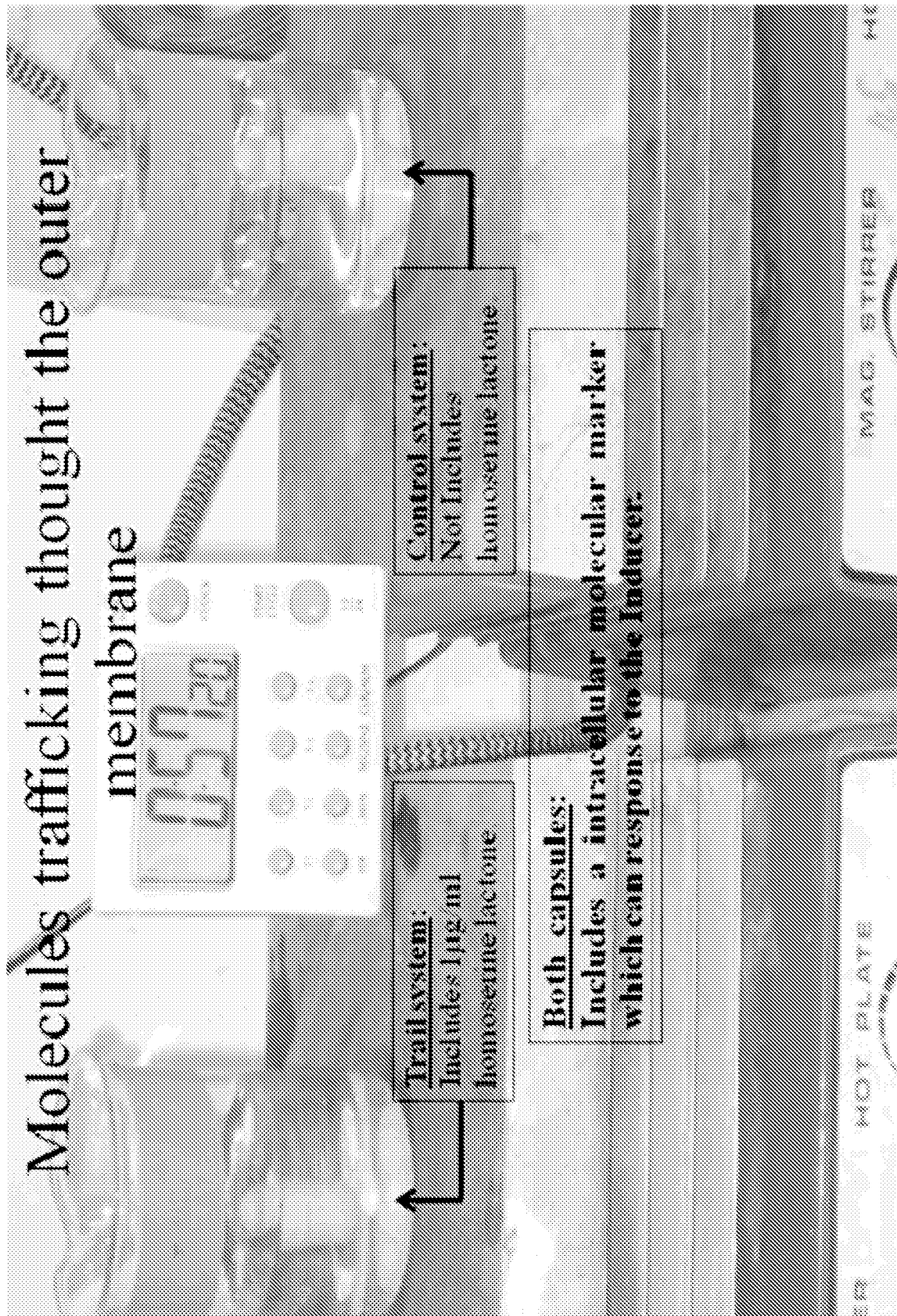


FIG. 15

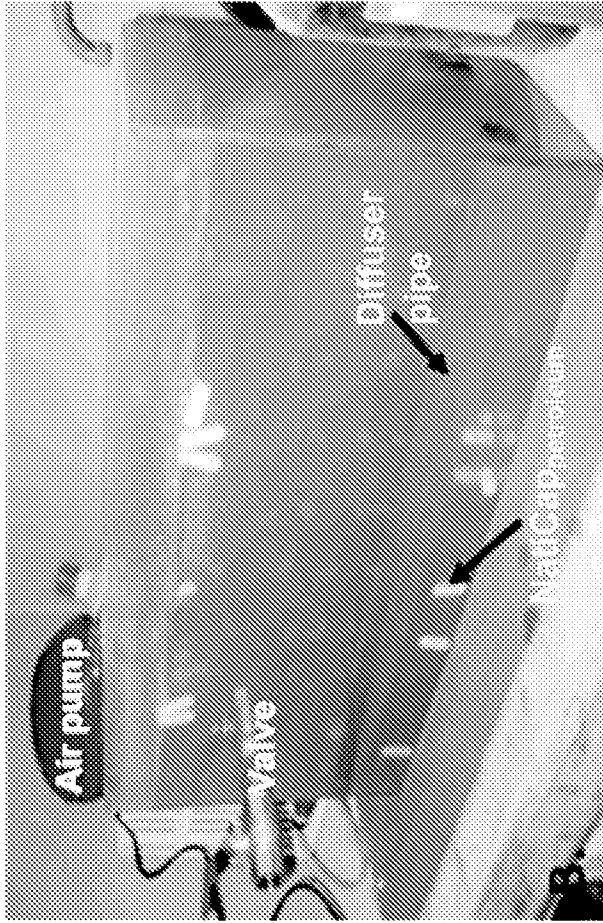


FIG. 16B

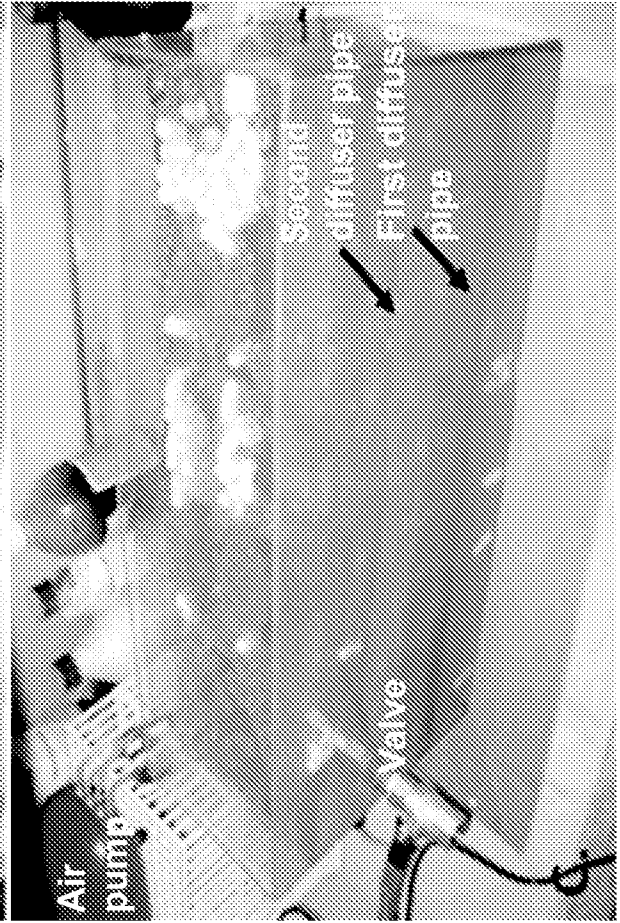


FIG. 16C

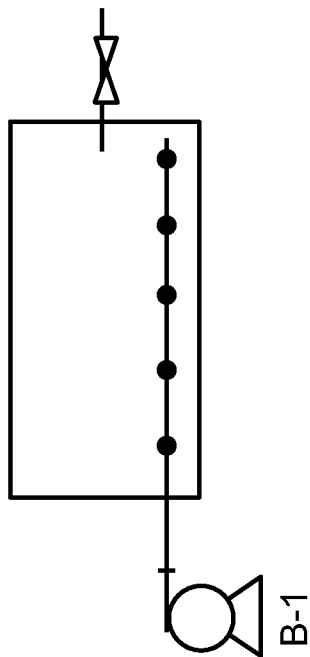


FIG. 16A

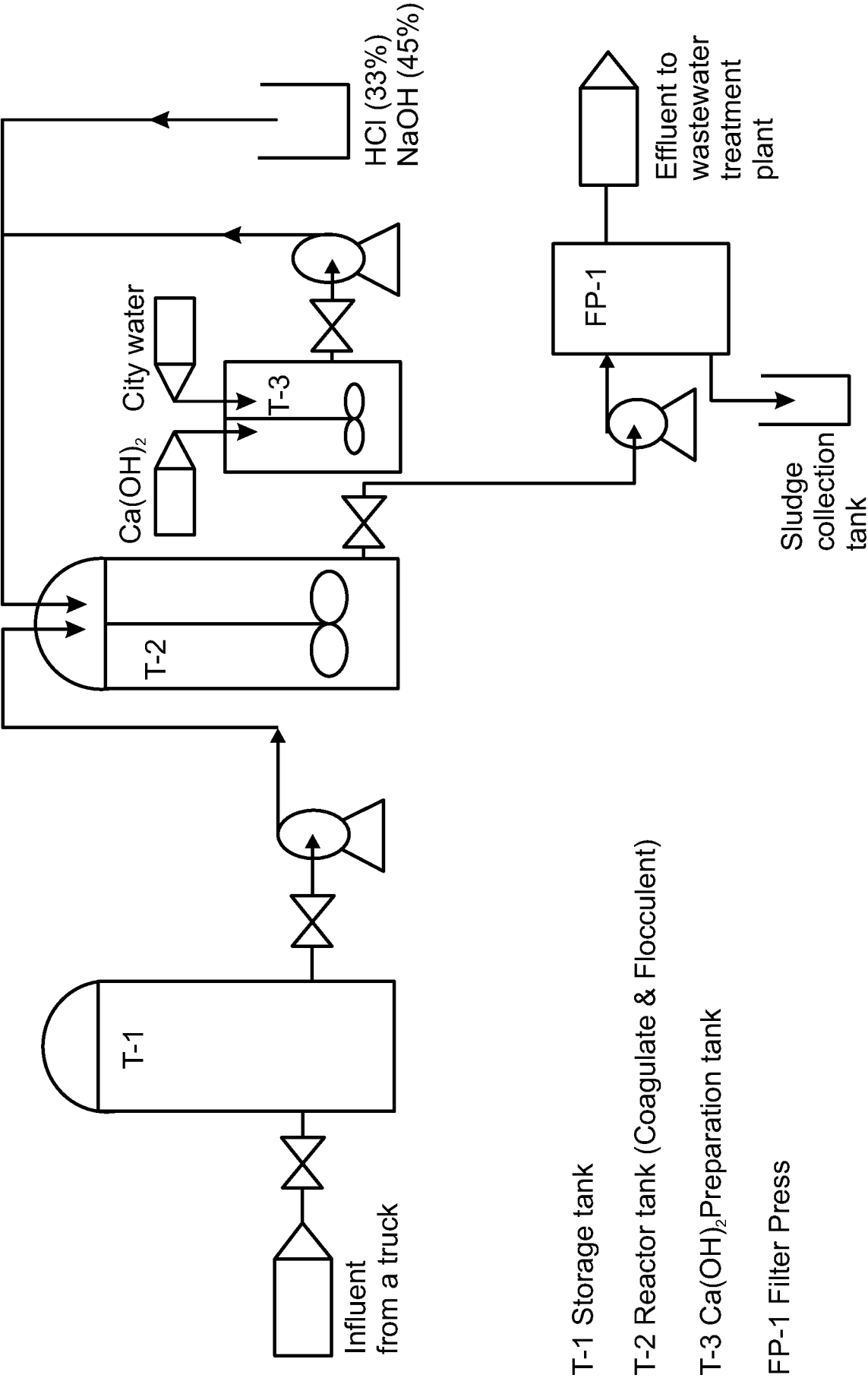


FIG. 17

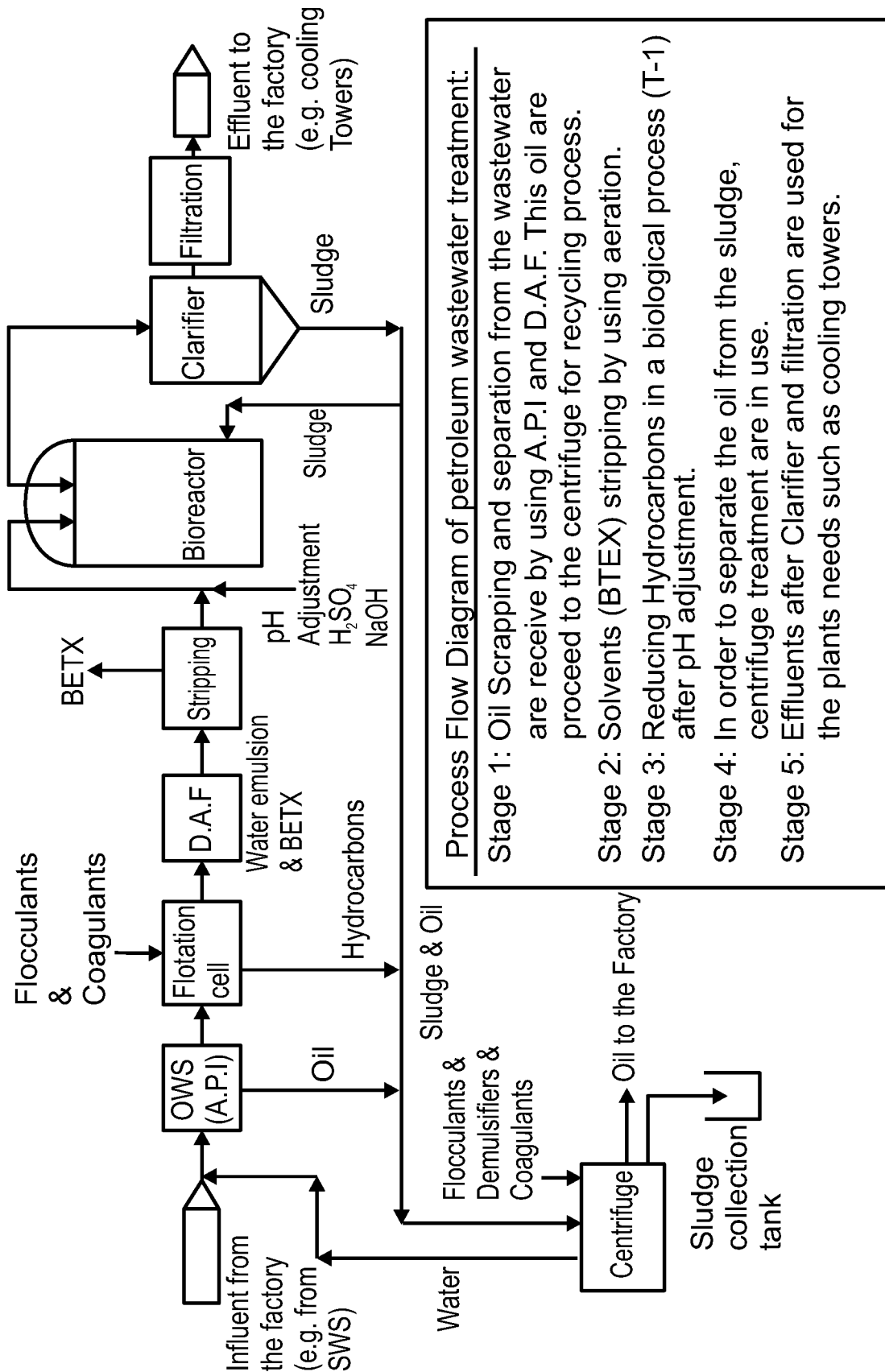
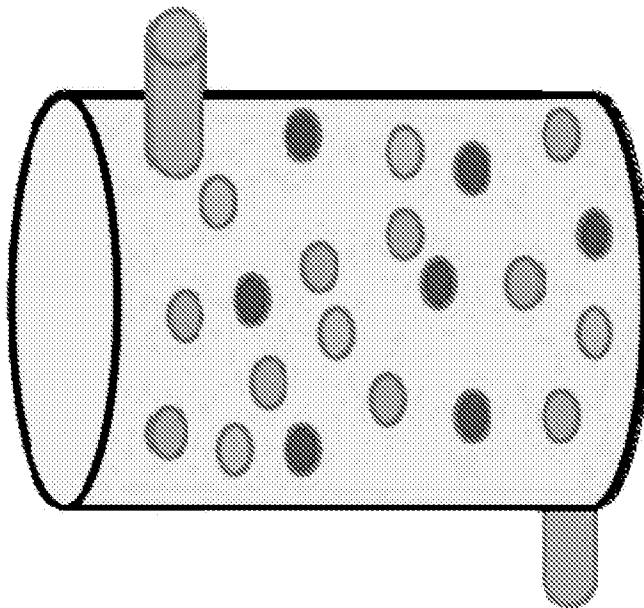


FIG. 18

FIG. 19A



- Bioreactor with culture type A
- Bioreactor with culture type B
- Bioreactor with culture type C

FIG. 19B

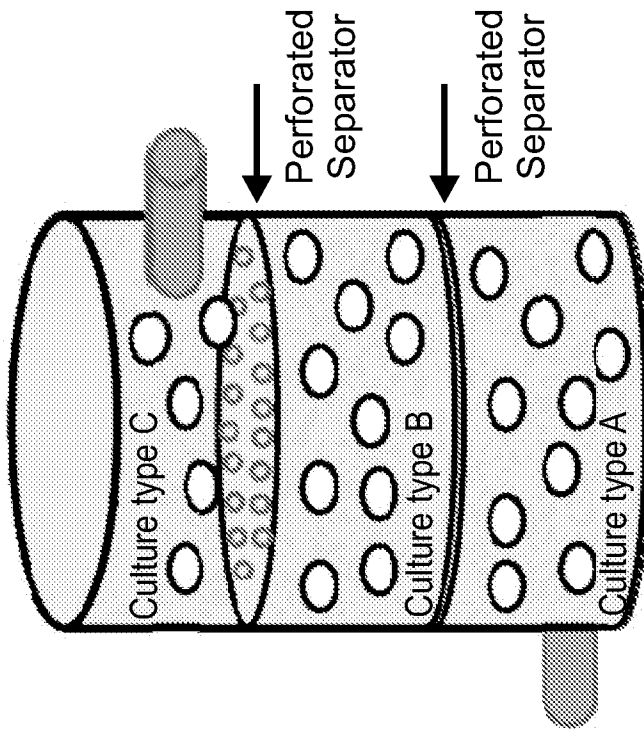


FIG. 20

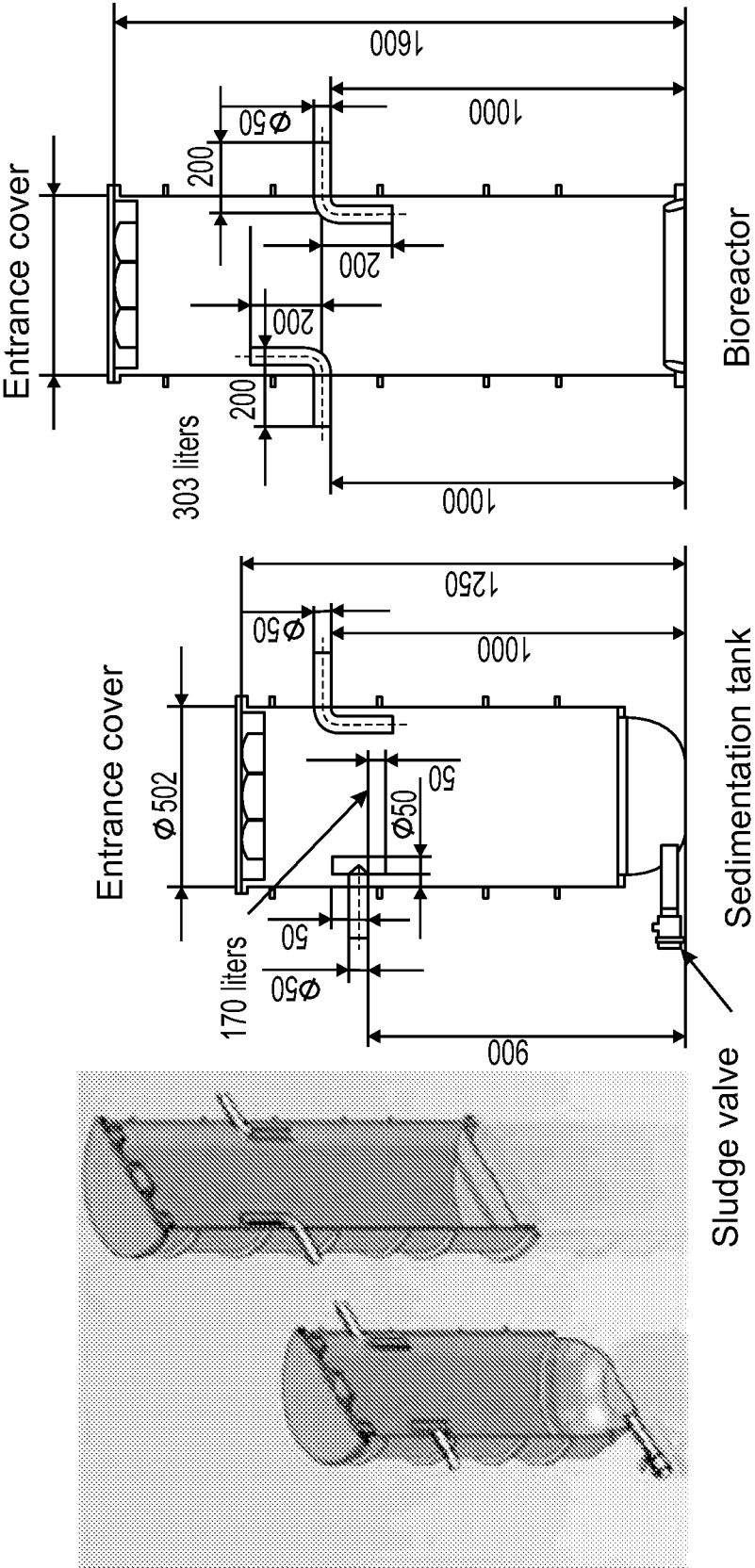


FIG. 21A

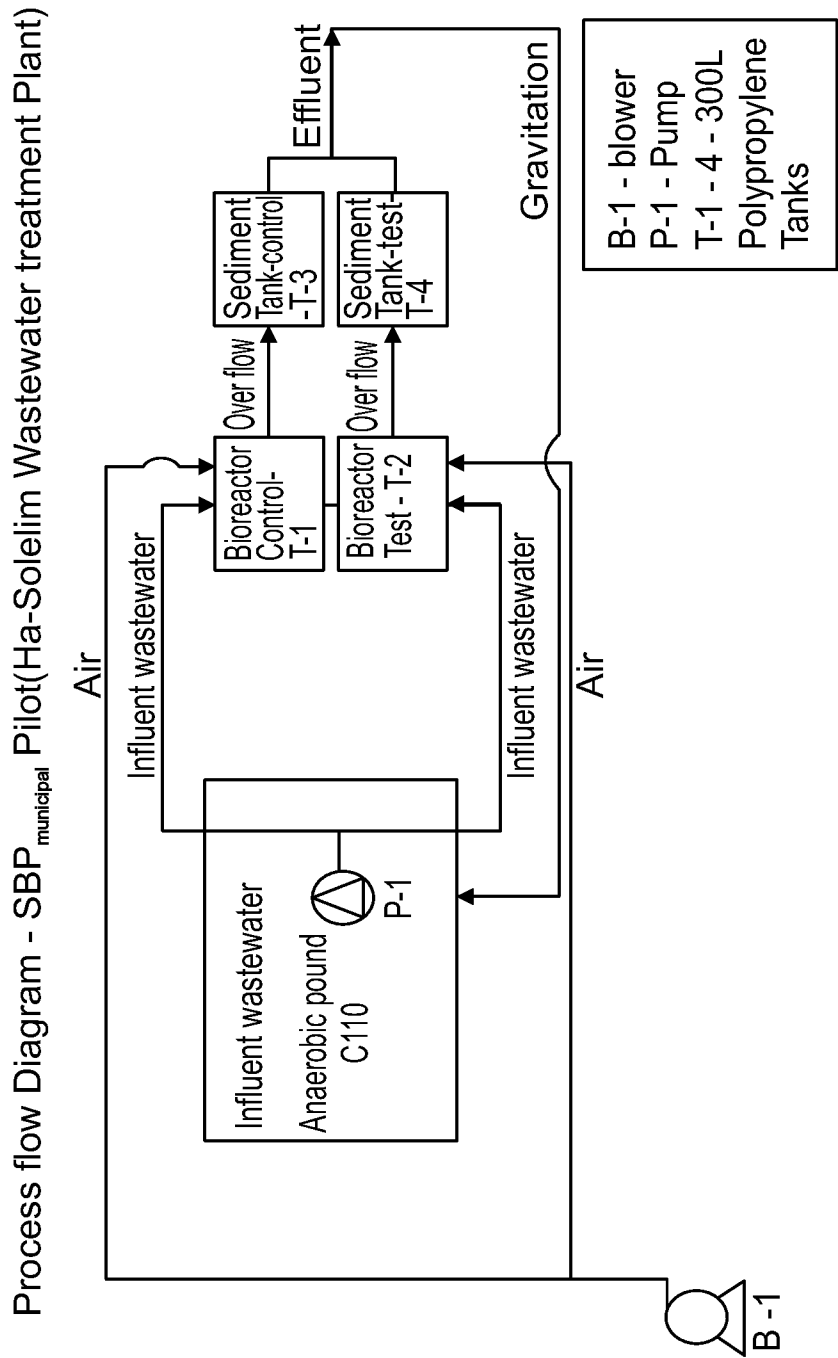


FIG. 21B

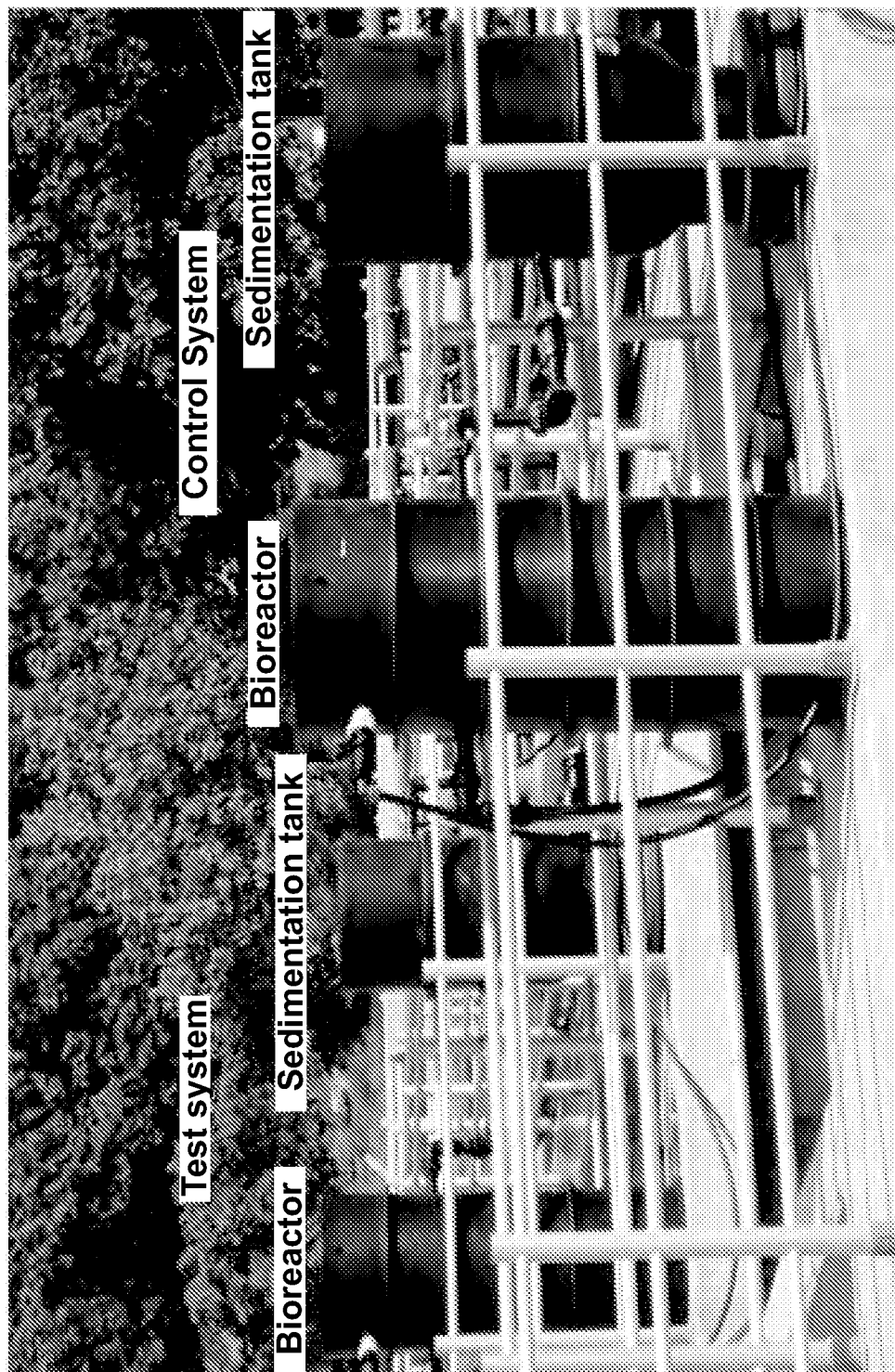


FIG. 22B

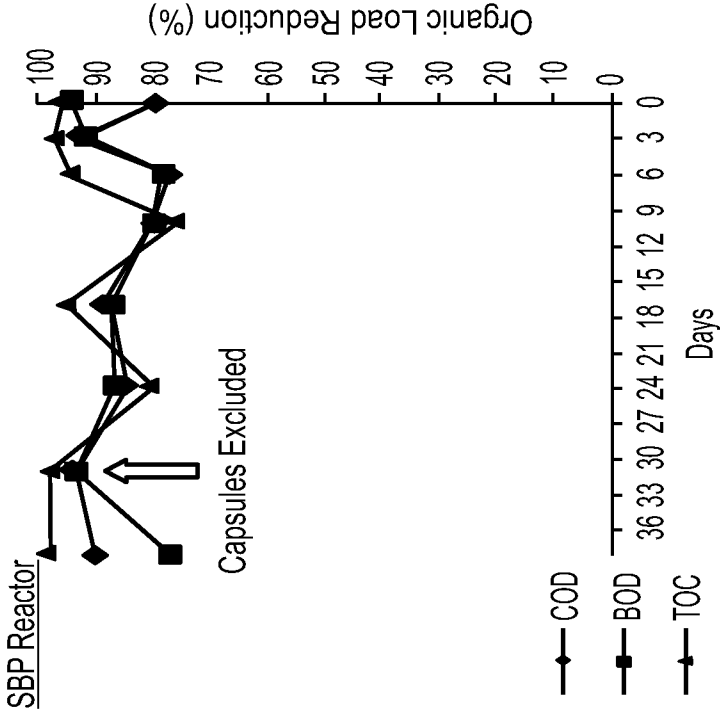


FIG. 22A

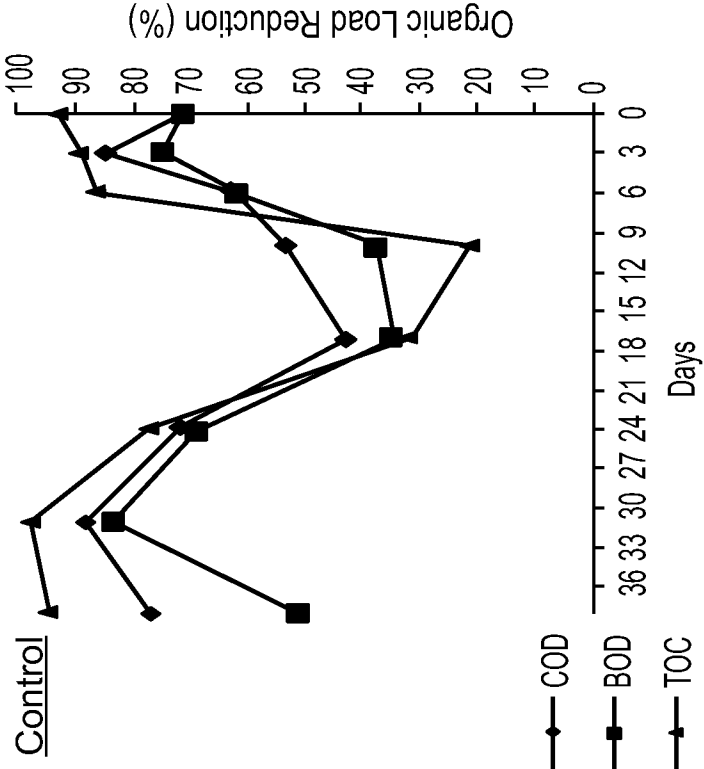


FIG. 23A

FIG. 23B

FIG. 23C

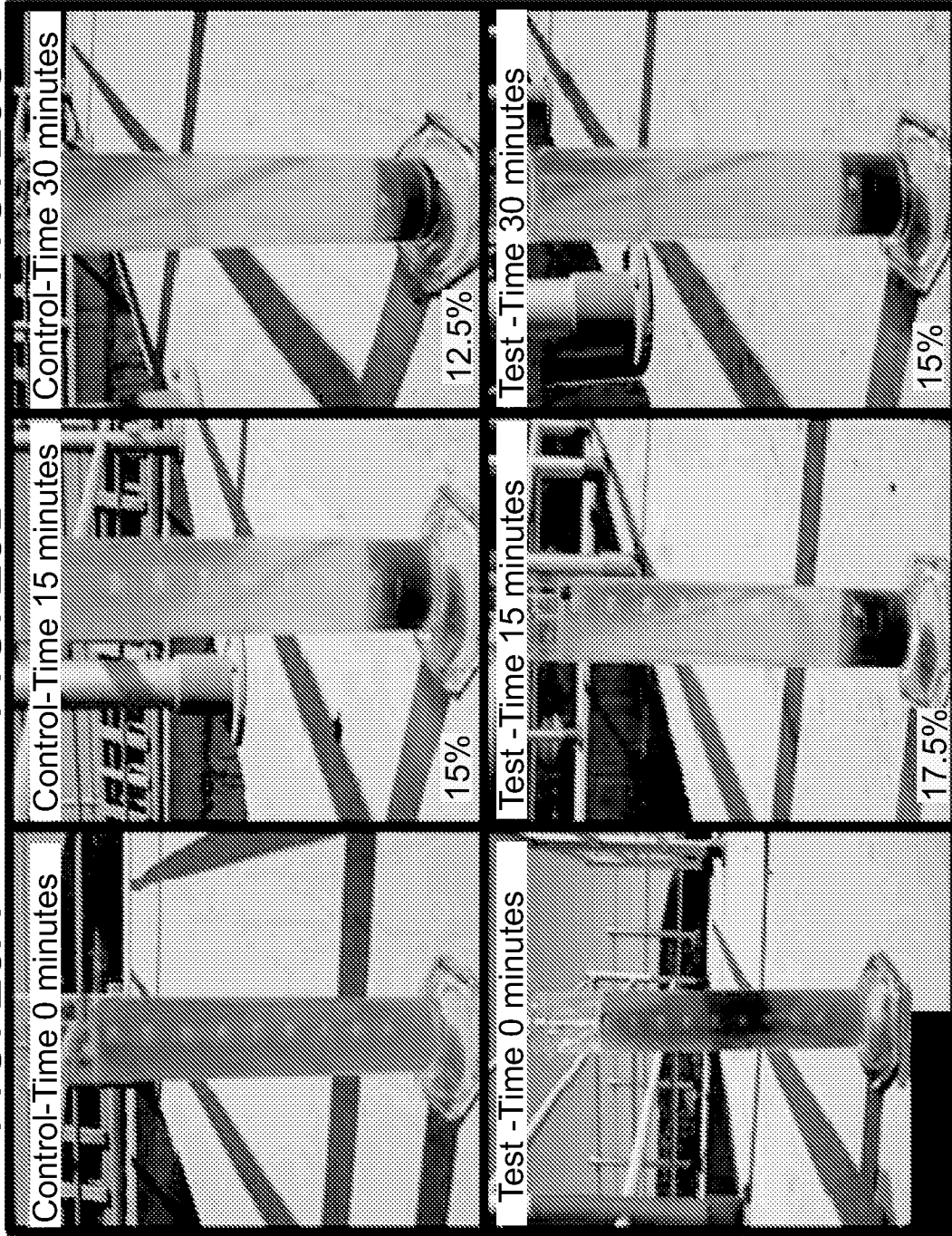


FIG. 23D

FIG. 23E

FIG. 23F

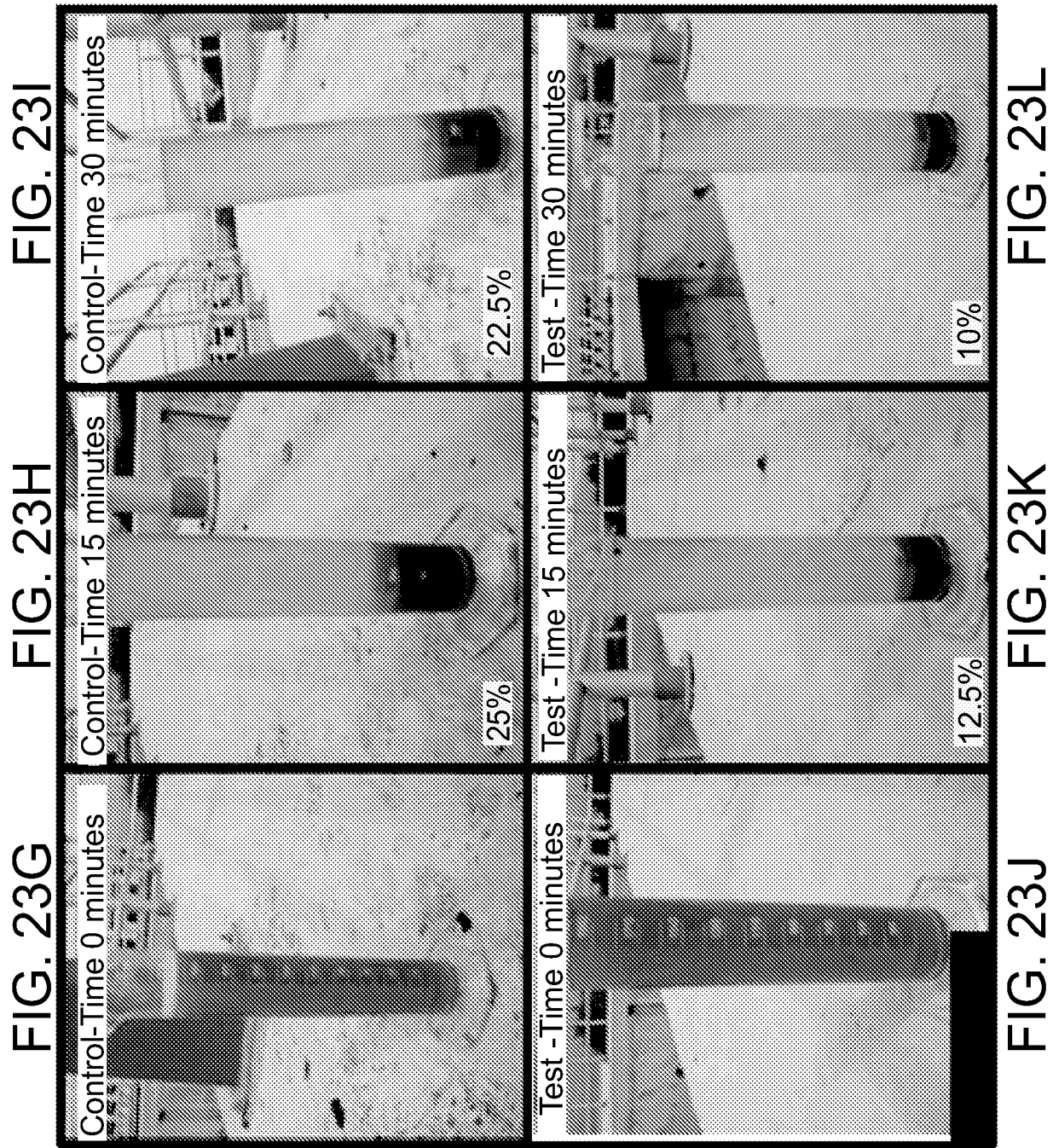


FIG. 24B

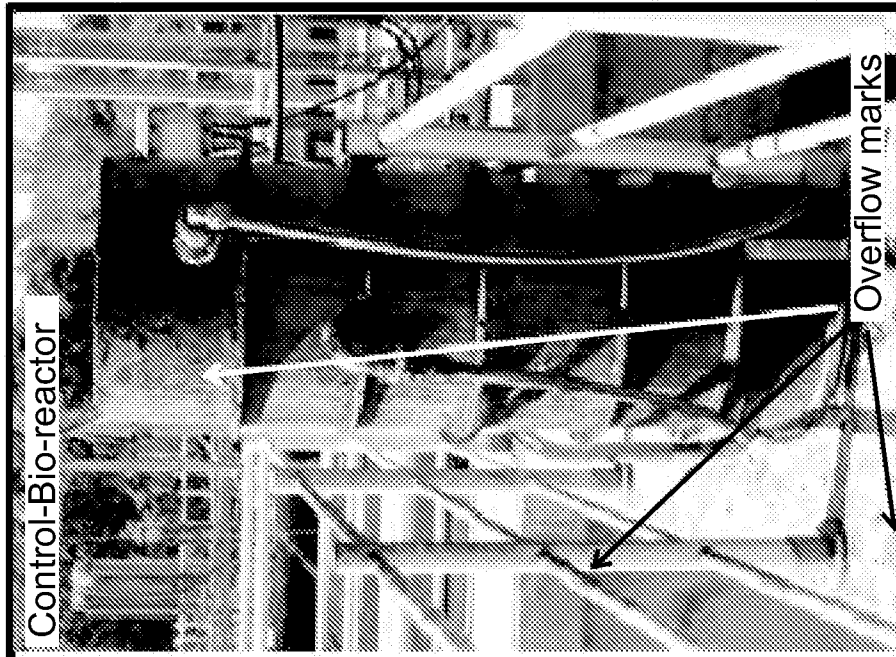


FIG. 24A



FIG. 24D

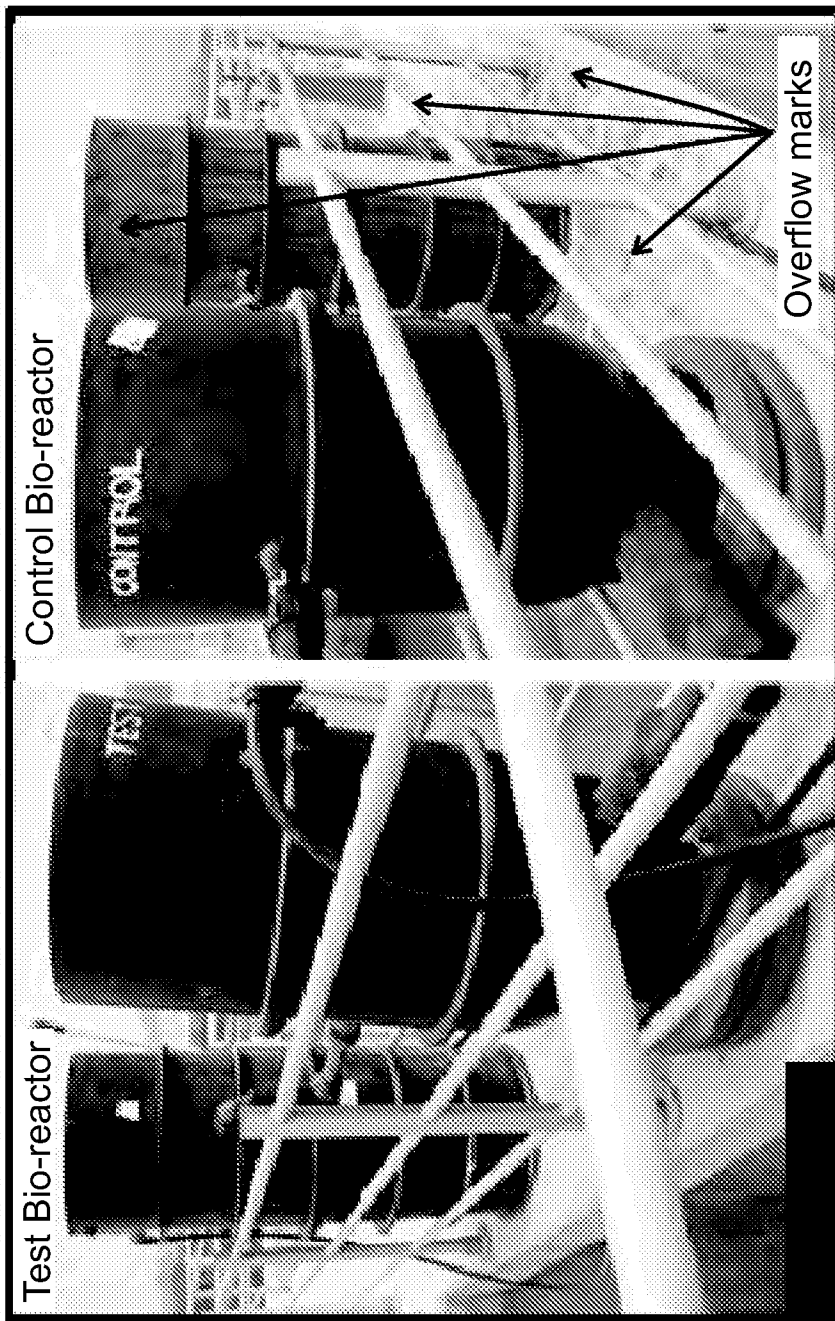


FIG. 24C

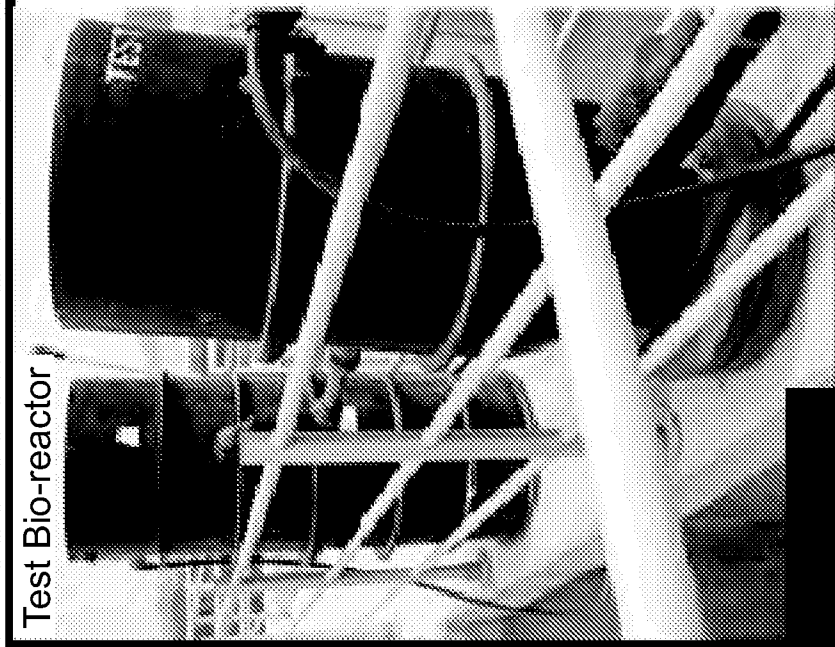


FIG. 25B



FIG. 25A



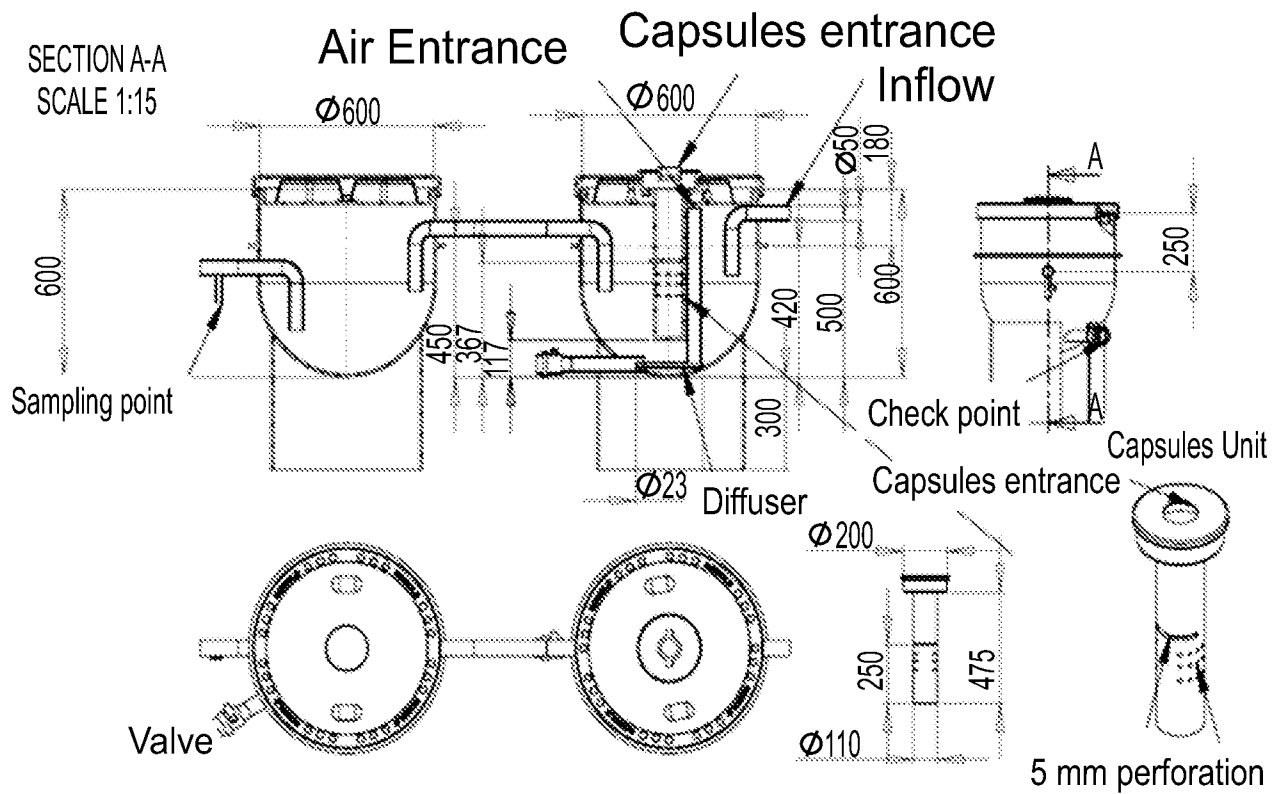


FIG. 26A

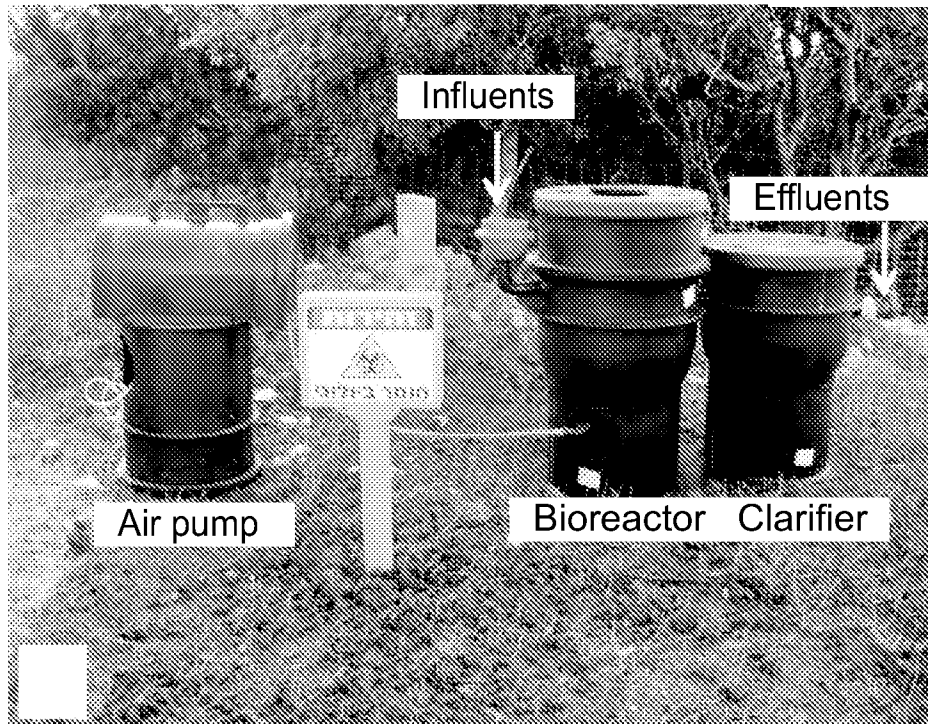


FIG. 26B

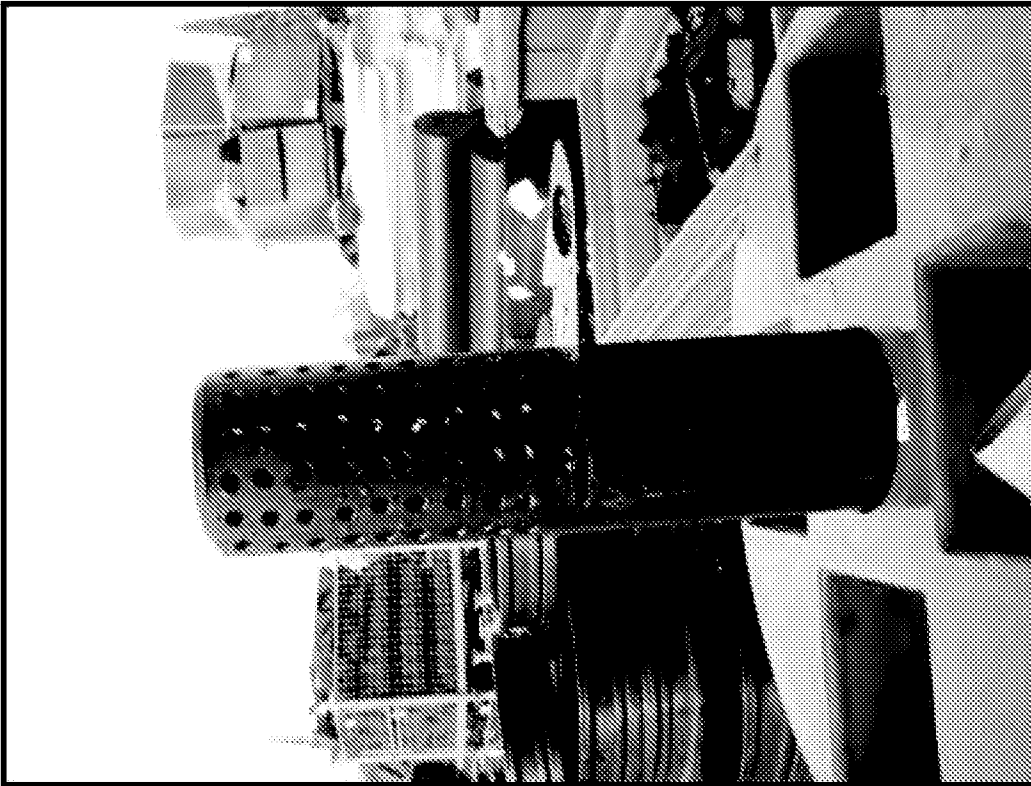


FIG. 26C

FIG. 27B

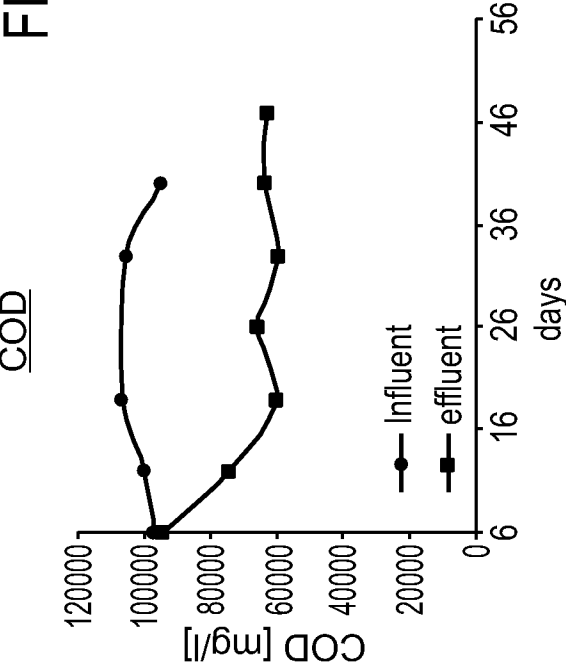


FIG. 27D

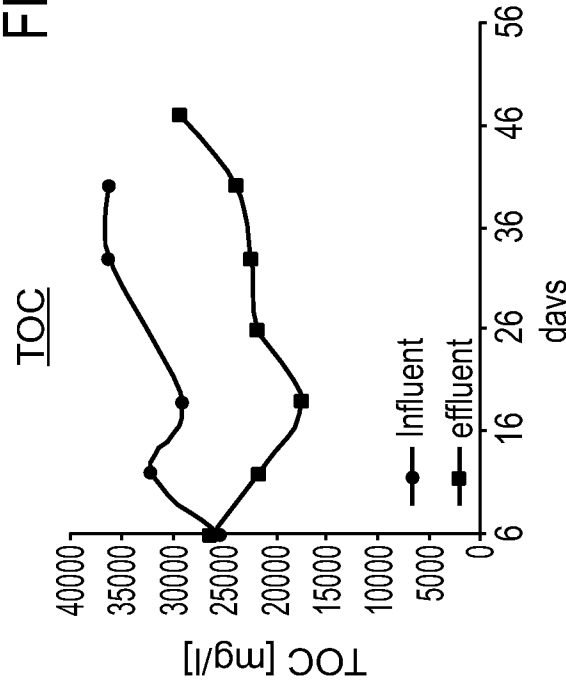


FIG. 27A

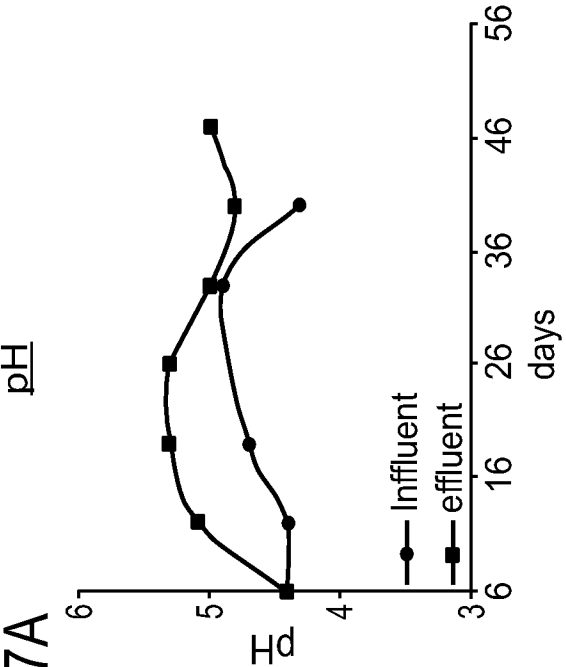
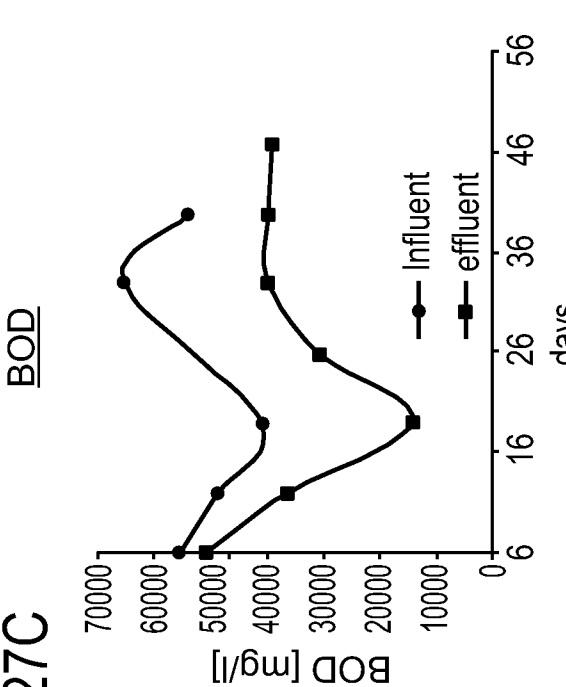


FIG. 27C



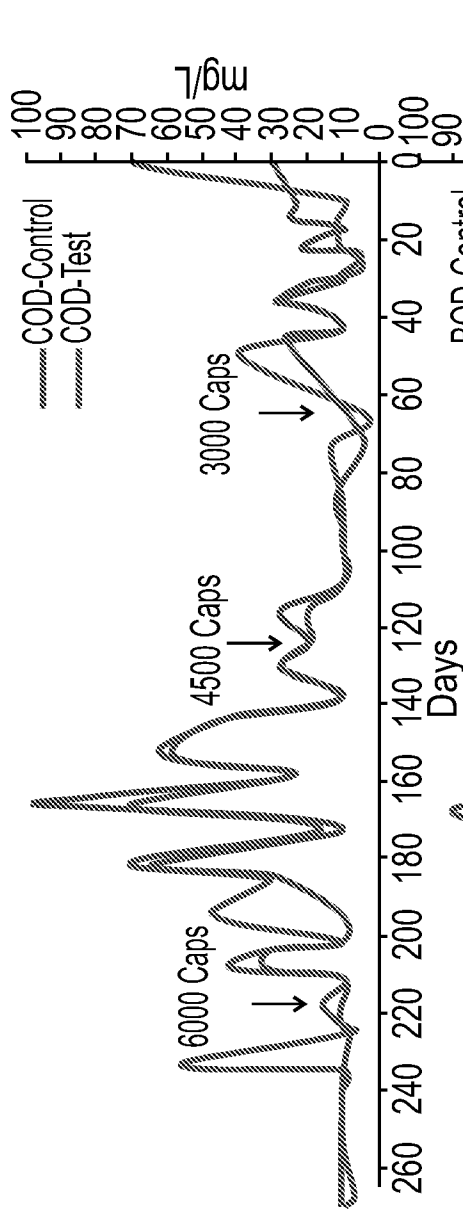


FIG. 28A

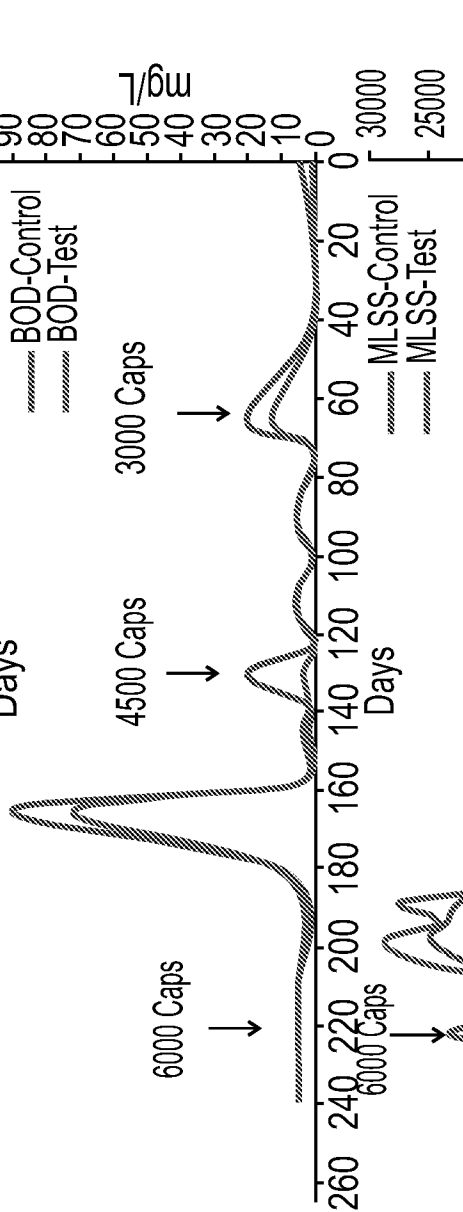


FIG. 28B

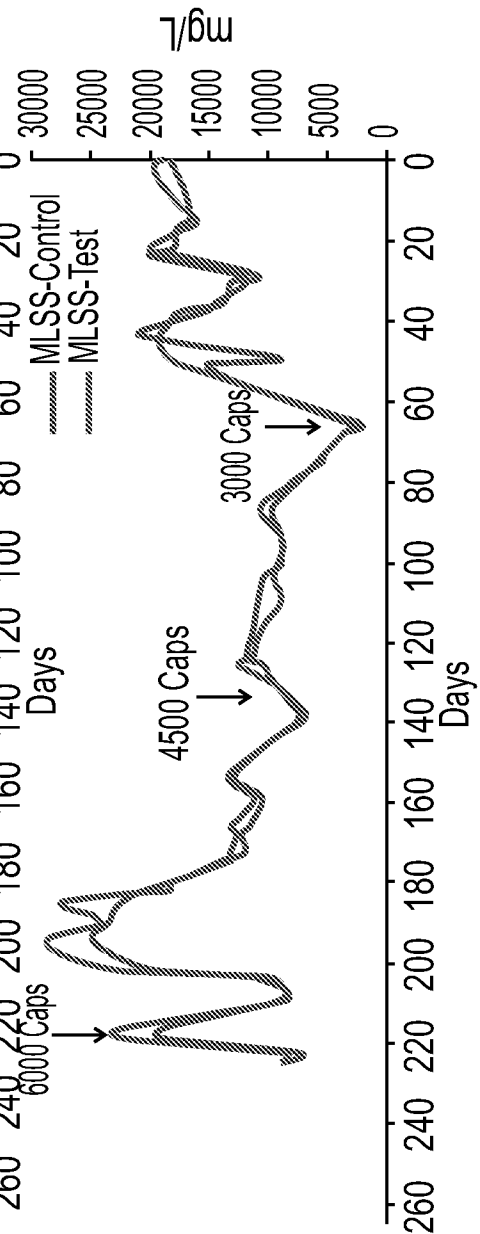


FIG. 28C

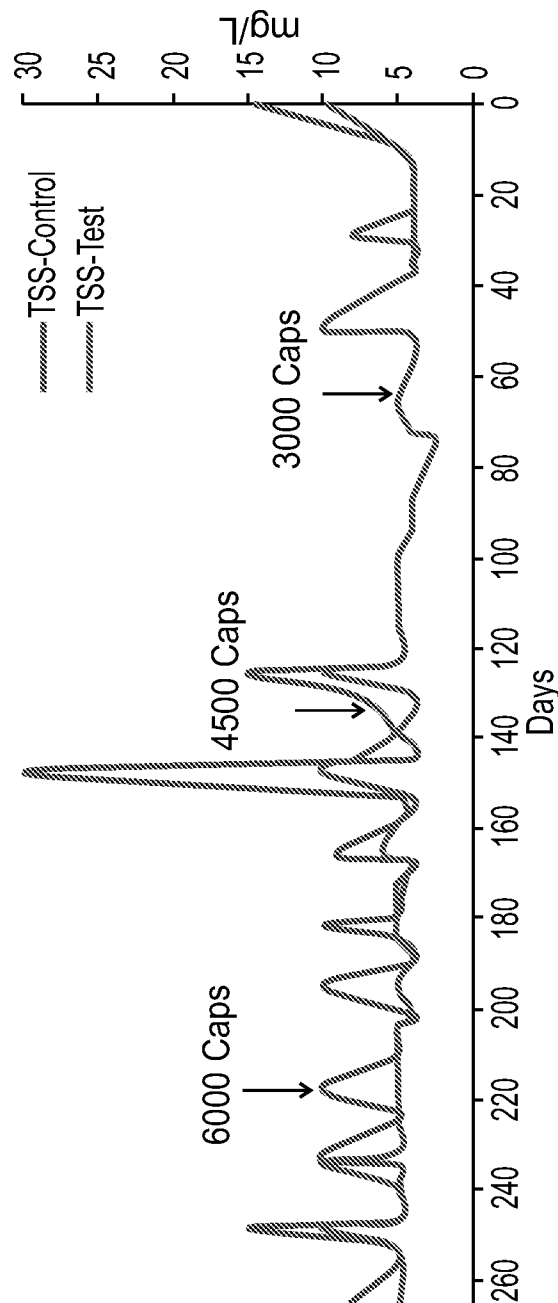


FIG. 28D

FIG. 29B

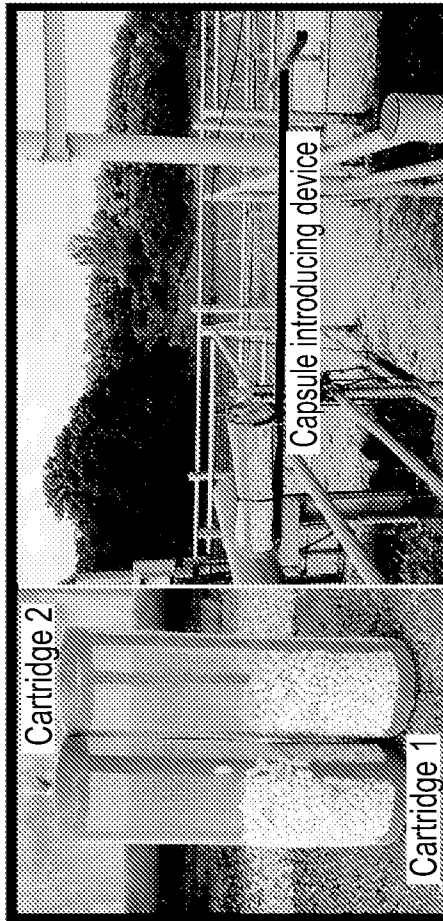


FIG. 29C



FIG. 29A

FIG. 30B

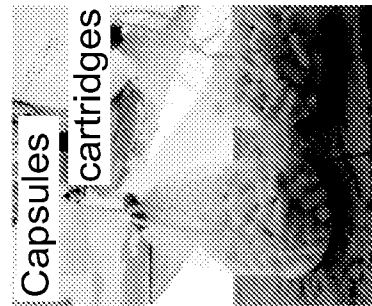


FIG. 30C

FIG. 30A



FIG. 31A

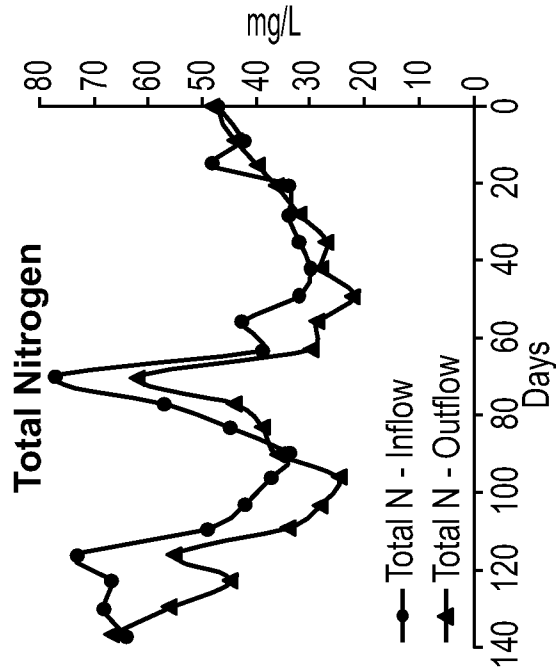
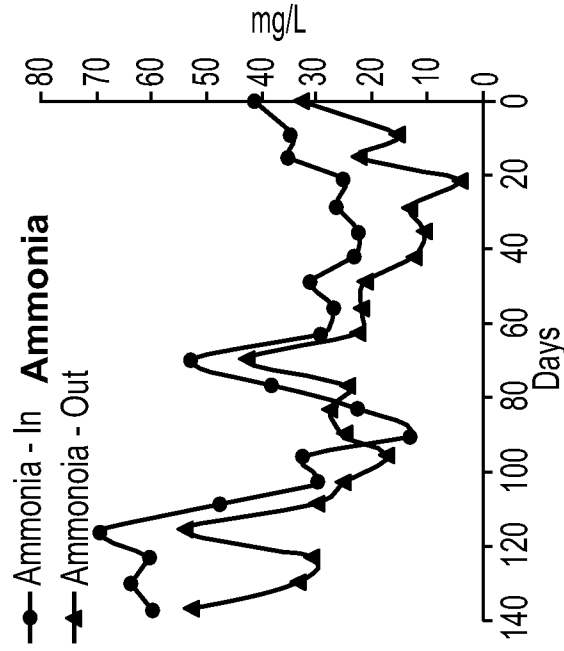


FIG. 31B



Total Nitrogen Kj

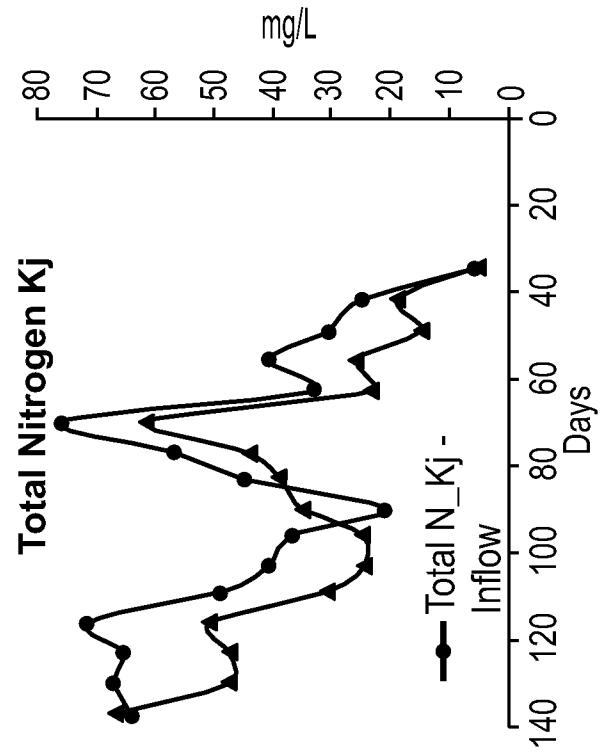


FIG. 31C

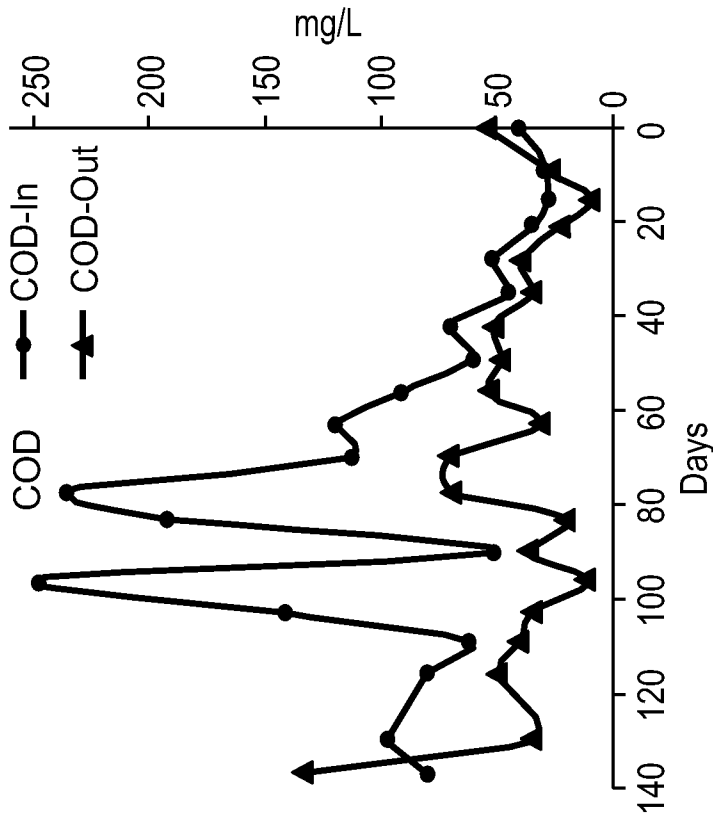


FIG. 32B

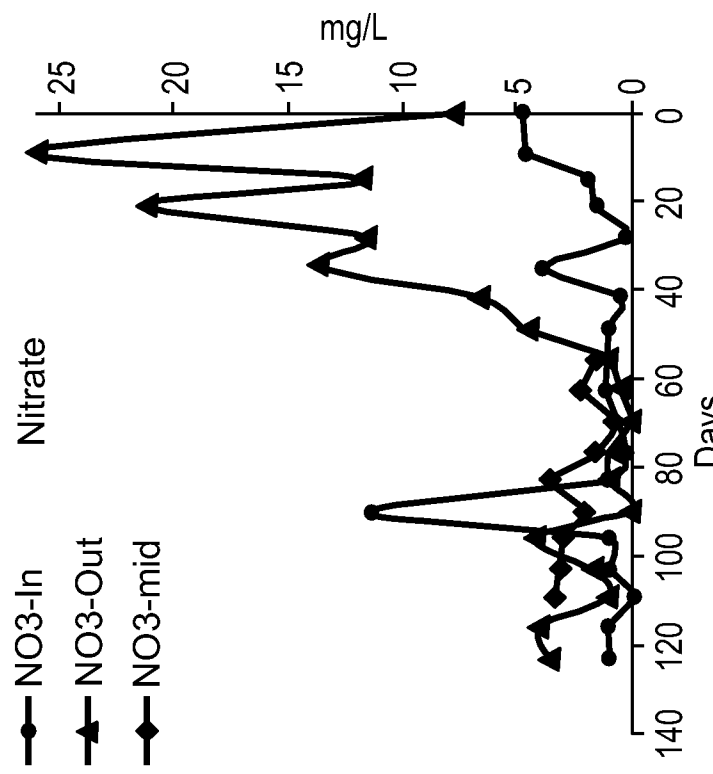


FIG. 32A