

Evaluation of Microbiologically Induced Corrosion on the life span of protective STOPAQ wrapping bands

Results of the evaluation of the hypothesis of: "NO nitrogen NO bacterial growth".
Literature Search, Experimental measurement and Screening tests
of all ingredients in the coating product on nitrogen content.

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October 2003.

**Evaluation of Microbiologically Induced Corrosion on the life span
of protective STOPAQ wrapping bands**

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Contract: Research project: Stopaq Wrapping band.
Project code: 030620

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SUMMARY

Subject:

Metal oil and gas transfer lines need to be protected by coatings to enhance their life span. Micro-organisms like bacteria, algae and fungi may accelerate the corrosion of metal tubes. For growth of microbial organisms the environment should be favourable, while essential nutrients are required by the microbes to expand.

Target:

Stopaq Europe B.V. initiated the evaluation of microbial attack on its Stopaq wrapping band, as a request of several customers. The Stopaq system has been developed strategically on the basis of a 3-layer water repellent and nitrogen free formula to inhibit (microbial) corrosion.

Previous test programs during the application of Stopaq showed that the wrapping band has excellent protection properties. The wrapping band is resistant to corrosive conditions and chemicals. High quality results were also obtained during the certification tests of KIWA. (e.g. strength, saponification value, UV-stability, temperature limits, adhesion, safety and environmental requirements, etc.; see section 2).

Microbiologically induced corrosion (MIC) is never observed during the several field tests. Since the MIC subject is not systematically investigated this research will emphasize the stability of the wrapping band for microbiological deterioration.

Research:

The hypothesis of: "no nitrogen, no bacterial growth" is evaluated.

The lack of nitrogen in the Stopaq wrapping band under anaerobic conditions is studied.

A literature search is done, instrumental measurements and nitrogen analysis on all ingredients of the formula and bacterial tests are performed.

The results of these studies are evaluated and presented in this report.

Conclusion:

For the Stopaq wrapping band application a large life span may be noticed, because microbiologically induced corrosion (MIC) of metal transport tubes is improbable.

The conditions for the growth of micro-organisms are very unfavourable:

- no water is available
- no nitrogen is available for bacterial growth
- the wrapping band is produced under sterilising condition (high temperature)
- the basic conditions (pH=8) of wrapping band prevents the development of SRB
- the self-recovering ability of the permanent viscous-elastic fluid material enhances the protection properties (*no cracks during long period of time*)

Microbial growth at the interface of the metal tube and the wrapping band is practically impossible, based on this study and on the empirically results of two performance studies during a period of 7 years.

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1. Introduction

In the context of the sustainability (life span) of organic protective coatings the question arises if a product will be biologically proof. In this matter the company of Stopaq Europe b.v. has asked CS Aspa University Laboratories to evaluate the Stopaq coating.

As a matter of fact the question of possible deterioration of the Stopaq wrapping band by bacteriological attack is due to the explicit and urgent request of several customers of Stopaq (e.g. NV Gasunie Netherlands: joint venture of Shell & Exxon) and the certification institute KIWA at Rijswijk.

It is known from literature that several publications handle the aggressiveness of micro-organisms and their emission of corrosive components on protective coatings (lit. 1,2,3 and 4). Also soil aggressiveness (lit 2) and the impact of sulphate reducing bacteria (SRB; lit. 3,4) may damage protective coatings including the transport tubes. The SRB are able to reduce sulphate to sulphide, which latter component may attack and dissolve the metal surface.

Because the Stopaq coating has been developed strategically on the basis of a nitrogen free formula, bacterial growth is in principle impossible.

In this report the hypothesis of "no nitrogen, no bacterial growth" is investigated. Literature is studied and nitrogen analysis is applied on all ingredients of the Stopaq formula.

The experimental results in combination with the theoretical and published data are discussed and evaluated in the context of microbiologically induced corrosion (MIC) for the Stopaq wrapping band (see section 8).

The final experiences are summarized in the conclusion (section 9).

2. Application of the protective Stopaq wrapping band .

Stopaq Europe at Stadskanaal, The Netherlands, produces a wrapping band for corrosive protection of (metal) oil and gas transfer lines. The Stopaq wrapping band is coiled around the tube and will stick to the surface (adhesion), preventing exposure to water and oxygen.

The outside layer of the wrapping band is constructed of a poly ethylene film (LDPE). The wrapping band is finally taped with a strong PVC adhesive band (total thickness 3.0 mm.) Next the total tube with coatings is buried in soil. This procedure assures that the coating is tightly pressed to the wall of the transfer line, expelling any airbubbles. Due to the fluidity of the Stopaq material all irregular shapes and pores in the metal are filled up.

Removal of the product shows only cohesive fractures, which means that the adhesion strength of the wrapping band to the metal is much larger than the internal cohesion strength. The final result of the Stopaq wrapping band application is always under anaerobic conditions, because the coating is tightly bound by adhesive strength to the protected tube.

(adhesion and surface penetration due to the permanent fluidity of the polymeric material)

Several tests of the firm Stopaq Europe and the client users, confirm these statements, after application of the wrapping band in practice.

In figure 1 an example is shown of the use of Stopaq for protection of oil transfes lines.

Figure 1. Protection of a gas transfer line with Stopaq wrapping band CZ H and PVC tape.
(Green part: Stopaq wrapping band with outside PE layer; Black part: PVC coated tube).



The Stopaq wrapping band has been investigated in the past in field tests for many times and during long periods of time (lit. 5). The aspects which have been tested are summarised:

- Visual examination
- Holiday detection (ASTM G62)
- Chemical resistance tests
- Impact resistance tests
- Electrochemical impedance measurements
- Cathodic protection/disbondment tests
- SEM and EDS analysis (electron microscopy; see section 6)
- Temperature limitation tests.

From these several previous test programs during the application of Stopaq it was concluded that the wrapping band has excellent properties. High quality specifications are obtained for electrochemical (cathodic protection), chemical corrosive resistance and self-recovering conditions (lit. 6,7). The wrapping band is resistant to corrosive chemicals and acids. Good results were also obtained during the certification tests of the certification institute KIWA (e.g. strength, saponification value, UV-stability, temperature limits, adhesion, safety and environmental requirements, etc. (lit.15). Other experiments reveal that the wrapping band is stable in acidic environments and that no water will diffuse when inorganic salt crystals are present at the surface layer (Mid East applications). In many cases several coatings deteriorate after a while from expanding salt crystals due to the adsorption of water from osmotic pressure. The wrapping band, however does not lose its (adhesive) protection specifications. *(no osmotic pressure due to permanent adhesive bond).*

The wrapping band application is hermetically closing the tube from the outside environment by adhesion of the sticky product to the tube. *(no permeation of water).* Accidental damages of the wrapping band are automatically restored due to the self recovering ability of the permanent fluid thermo elastic material.

After a field test of 7 years in acidic wet soil the Stopaq CZ wrapped pipe-wall remained in excellent condition, with no measurable corrosion (lit. 8). A simultaneous experiment was done within the facilities of Stopaq Europe b.v. by plunging a wrapped tube in aerated water at ambient temperature. The coating is inspected annually since 1996. During a period of 7 years no microbial activity is observed (see figure 2).

This report will only emphasize the research of microbiologically induced corrosion.

Figure 2. Released parts of coating after 7 years.
(Metal parts are blanc: no corrosion could be observed)



3. Microbiologically Induced Corrosion.

Corrosion can be defined as the transformation of a metal from an insoluble metallic state to a soluble state. Well known corrosion processes are galvanic and electrolytic types, but also microbes and their emission products may attack metal tubing.

During microbiologically induced corrosion (MIC), electrons are transferred from an anode (metal; $\text{Fe} \gg \text{Fe}^{2+} + 2\text{e}$) to a cathode (environment). Bacteria may transfer these electrons by specific catalytic enzymes while they gain energy from this process. Metallic corrosion organisms are sulphate reducing bacteria (SRB), iron bacteria and methanogenic bacteria.

For bacterial growth always nutrients are required:

- most important is water and a carbon source to provide energy and to build elemental cell material, but also nitrogen is required for reproduction of bacterial cells.
- less important but still essential are some elements like phosphorus, magnesium, calcium, potassium and sodium
- at last minor trace elements are necessary for favourable conditions

Principles of microbial corrosion bacteria.

Micro organisms like bacteria, algae and fungi may accelerate the corrosion of metal transfer lines. Under anaerobic conditions the microbes may produce emission products like volatile fatty acids (VFA) from organic substrates or sulphides from sulphate. It is well known that acids and sulphides are able to oxidise metals, hence corroding the metal material.

All internal bacterial reactions take place in the water environment of the cell.

For these micro organisms the environment should be favourable. At first ambient temperature ($25^\circ \text{C} \pm 20$) is required and furthermore nutrients are necessary for the microbes; e.g. carbon, nitrogen, phosphorus, oxygen, sulphur and hydrogen.

Additional elements in trace amounts are required to create ideal conditions; e.g. potassium, magnesium, calcium, iron, copper, zinc, cobalt and manganese.

Microbes may exploit the oxidation reactions of inorganic material (iron) for energy supply. The life cycle of sulphur oxidising ($\text{SO}_4 > \text{H}_2\text{S}$) and iron oxidising ($\text{Fe} > \text{Fe}^{++}$) bacteria is based on this principle. Other bacteria obtain their energy from the oxidation of organic molecules like carbohydrates, alcohols and cellulose.

Their energy is stored as adenosine triphosphate (ATP) and will become available to drive several stepwise biochemical reactions in the cell, catalysed by enzymes. The stepwise removal of the electrons is carried out by various carriers in the aqueous solution of the cell.

If the terminal electron acceptor is:

- i. - atmospheric oxygen the process is defined as aerobic respiration
- ii. - an organic molecule it is called fermentation
- iii. - an inorganic component (no oxygen) the process is anaerobic

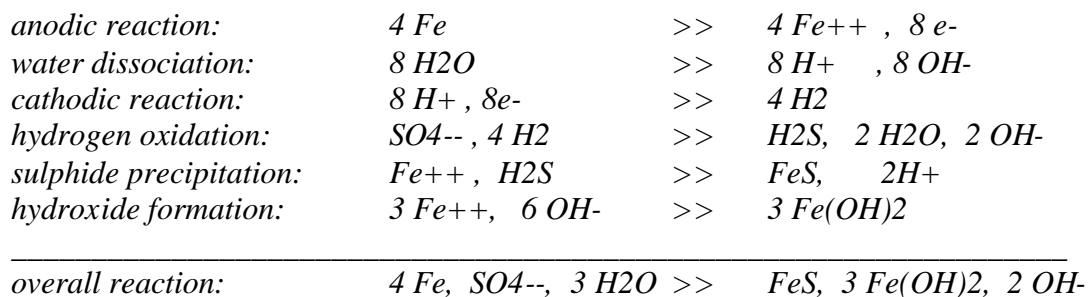
Anaerobic sulphate reducing bacteria (SRB) may reduce sulphate and the resulting sulphide product may corrode metals.

Sulphate Reducing Bacteria (SRB).

Some examples of sulphate reducing bacteria are Desulfovibrio and Desulfotomaculum. In many cases SRB derive their carbon energy from organic material, but they also have the ability to gain energy from the oxidation of sulphate to sulphide. Their enzyme hydrogenase can even obtain energy from the oxidation of molecular hydrogen ($H_2 \gg H^+$).

The anaerobic corrosion of iron is of great importance. It diminishes the life time of steel and iron material. When iron is immersed in water it releases iron ions (Fe^{++}). The result is that the iron (Fe) is charged by remaining electrons. In the absence of oxygen the electrons left on the iron reduce protons, from the dissociation of water (if available), to hydrogen.

The hydrogen protects the iron surface from further dissolving, unless a SRB bacterium is present, which is capable to convert (oxidation of) hydrogen (SRB with hydrogenase). In the next reaction scheme the probable mechanism (not exactly known) is shown: (lit. 4)



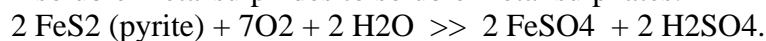
Thermodynamic calculation results in an enthalpy contribution for the SRB of approximately 400 Joules for this specific reaction.

According to this reaction scheme under anaerobic conditions, water is required for the production of hydrogen. Next when sulphate and SRB are present the bacterial attack may continually deteriorate the iron surface (pH range 4-8; temp.range 10- 70 °C). From the overall reaction it is clear that hydroxide (OH^-) is formed, so in acid environment this reaction will be accelerated by the neutralising proton H^+ . Under basic conditions (pH > 8; excess of OH^- present) the reaction will be hindered.

In theory this "maintenance process" of the culture in acidic environment may slowly continue, but the population of SRB can only grow and reproduce if essential nutritional elements (e.g. carbon and **nitrogen**, etc.) are available.

Sulphate Oxidising Bacteria.

Sulphate oxidising bacteria (SOB) are slime forming aerobic organisms. Examples are Thiobacillus thiooxidans and Thiobacillus concretivorus. The microbes are capable to oxidise insoluble metal sulphides to soluble metal sulphates:



The pH may reduce to 1 or less with the consequence of attacking metals.

In the symbiotic combination of SOB with SRB the sulphates may be converted to sulphides, as described above.

4. Theoretical approach

The hypothesis in this particular case is: "no nitrogen, no bacterial growth".

It means that bacterial growth is impossible at the boundary of the protective organic coating and the metal coating wall, if there is a lack of nitrogen.

Generally speaking for living organisms the first need is a moisty (water) environment and secondary a carbon compound feed (energy) is a necessity. But for the life on earth also the nitrogen cycle is of fundamental importance.

In this chapter 4 an approach is made to highlight the need of the essential nitrogen element for bacterial growth.

The following knowledge is based on three literature references: 9,10,11.

Proteins

Proteins consist of a chain of coupled amino acids. Since all amino acids contain a nitrogen atom, this element is essential for the construction of new amino acid molecules to reproduce proteins.

The number of known amino acids is over 100 but the most important ones (α -amino acids in proteins) for living cells are shown in table I, with their molecular weight and the percentage (% w/w) of nitrogen.

Table I. Amino acids.

Amino acid	Abbreviation	Molecular formule	Molecular weight	% N (in molecule) (w/w)
Glycin	Gly	C ₂ H ₅ O ₂ N	75	18.7
Alanin	Ala	C ₃ H ₇ O ₂ N	89	15.7
Valine	Val	C ₅ H ₁₁ O ₂ N	117	12.0
Leucin	Leu	C ₆ H ₁₃ O ₂ N	131	10.7
Isoleucin	Ileu	C ₆ H ₁₃ O ₂ N	131	10.7
Phenylalanin	Phe	C ₉ H ₁₁ O ₂ N	165	8.5
Tyrosin	Tyr	C ₉ H ₁₁ O ₂ N	181	7.7
Prolin	Pro	C ₄ H ₈ O ₂ N	102	13.7
Hydroxyprolin	Hypro	C ₄ H ₈ O ₃ N	118	11.9
Serine	Ser	C ₃ H ₇ O ₃ N	105	13.3
Threonine	Thr	C ₄ H ₉ O ₃ N	119	11.8
Cysteine	CySH	C ₃ H ₇ O ₂ SN	121	11.6
Cystine	Cys.SCy	C ₆ H ₁₂ O ₄ S ₂ N	226	6.2
Methionine	Met	C ₅ H ₁₁ O ₂ SN	149	9.4
Tryptophane	Try	C ₁₁ H ₁₁ O ₂ N ₂	203	13.8
Aspartic acid	Asp	C ₄ H ₇ O ₄ N	133	10.5
Glutamic acid	Glu	C ₅ H ₉ O ₄ N	147	9.5
Arginine	Arg	C ₆ H ₁₄ O ₂ N ₄	174	32.2
Lysine	Lys	C ₆ H ₁₄ O ₂ N ₂	146	9.6
Histidine	His	C ₆ H ₉ O ₂ N ₃	155	27.1
		Mean Value:		13.2
		Minimum:		6.2
		Maximum:		32.2

Based on the concept of living material all organisms contain amino acids. The amino acids are essential bricks in cell material. The DNA material is found in every reproducing cell.

Nucleotides

Nucleotides are organic components composed of nitrogen-containing units coupled to sugar and phosphate units. The natural occurring nucleotides contain nitrogen bases with pyrimidine, purine, pyridine, nicotinamide, adenine, guanine, cytosine, thymine or uracil. The nitrogen content of these naturally occurring bases is shown in table II.

Table II. Nitrogen bases in nucleotides and their mass percentage of nitrogen.

Nitrogen base	Formula	Molecular weight	% Nitrogen (w/w)
Pyrimidine	C ₄ H ₄ N ₂	80	35.0
Purine	C ₅ H ₃ N ₄	119	47.0
Pyridine	C ₅ H ₅ N	79	17.7
Nicotinamide	C ₆ H ₆ ON ₂	122	23.0
Adenine	C ₅ H ₅ N ₅	135	51.8
Guanine	C ₅ H ₅ ON ₅	151	46.4
Cytosine	C ₄ H ₅ ON ₃	111	37.8
Thymine	C ₅ H ₆ O ₂ N ₂	126	22.2
Uracil	C ₄ H ₄ O ₂ N ₂	112	25.0
	<i>Mean value:</i>	115	34.0
	<i>Minimum:</i>	79	17.7
	<i>Maximum:</i>	151	51.8

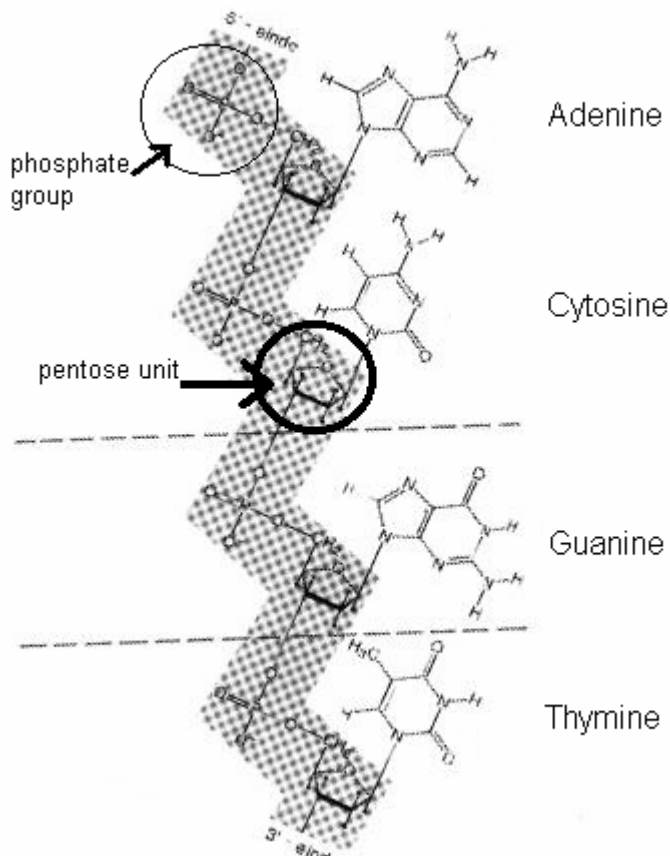
Nucleic acids

Nucleic acids are polynucleotides, long chain compounds consisting of repeating structural units called nucleotides (see figure 3).

Nucleic acids are very important, because they provide the genetic material of a cell, by directing the process of protein synthesis in all living material. There are two classes of nucleic acids: the RNA (ribonucleic acid) and DNA (deoxyribonucleic acid).

Both RNA and DNA are constructed by a pentose sugar, a purine or pyrimidine base and a phosphate residue. The macromolecules (giant molecules) that can be distinguished from each other by their base and sugar contents: in RNA the pentose sugar is ribose while in DNA the pentose sugar is 2-deoxyribose.

Figure 3. Small part of DNA-structure with 4 different nucleotides.



From table III it can be seen that DNA material from different species (sources) are quite similar in their structure of nucleotides; the sum of the 4 different ones is almost 100%. This may confirm the correctness of the approximation of calculation of the nitrogen content (see next paragraph).

Table III. Composition of DNA sources, relative to the nucleotides of figure 1 (lit.9).

<i>DNA source</i>	<i>% Adenine</i>	<i>% Thymine</i>	<i>% Guanine</i>	<i>% Cytosine</i>
Human sperm	31.0	31.5	19.1	18.4
Escherichia coli	26.1	23.9	24.9	25.1
Mycobacterium tuberculosis	15.1	14.6	34.9	35.4
Bacteriophage T2	32.6	32.6	18.2	16.6

Calculation of nitrogen content in nucleic acid.

Since nucleic acids contain many nucleotides, one may assume that the percentage of nitrogen in a nucleotide will be the same as in the nucleic acid.

The nucleotide is built by 1 mole pentose (M= 133), one mole of a nitrogen base (example: pyridine M = 79) and a phosphate group (M= 95).

The total mass of this reasonable example molecule is 307.

The nitrogen content of pyridine is 17.7 % , hence the overall nitrogen percentage in the total nucleic acid molecule in a worst case scenario is at least: $(79 \times 17.7 / 307)$ 4.5 % nitrogen.

More likely is to use the mean value of the molecular weight of the nitrogen base (M= 115) and the mean value of its nitrogen content (34.0 %), see table II.

The new calculation results in the mean nitrogen content of a nucleic acid, which is 12 % nitrogen $(115 \times 34.0 / (113+115+95))$.

DNA occurs in a constant amount in all body cells of a particular species.

Since DNA is occurring in cell nuclei as a constituent of chromosomes, where it serves to encode genetic data, the species need nitrogen for growth.

Which means that a lack of nitrogen may act like a biocide.

Proteins are constructed from many different (> 20) amino acids, while the nucleic acid DNA is mostly build out of 4 different nucleotides.

It has already been estimated that the nitrogen content of proteins is 13.2 % N (table I).

It may be concluded now that if living organic material consists of mainly nucleic acids and proteins their nitrogen content will be approximately: 12.5 % nitrogen. This statement is corresponding with the nitrogen content mentioned in several text books.

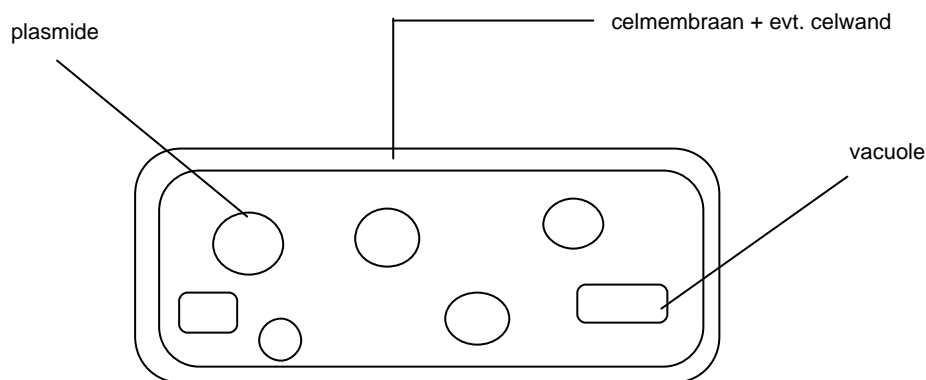
So to build new organic material for growth of an organism approximately 12 % of its dry weight is required to form new organisms.

Bacteria.

Bacteria are present everywhere in the environment, they can be in competition or in symbiosis with human targets. Bacteria are the most primitive forms of life but without them, human life would be impossible. The water containing one-cell organisms can reproduce very fast by cell-dividing. In contrast with other organisms the genetic material is not included in the membrane of the cell, but is spread over the whole cell in the so called plasmids. Plasmids are ring-shaped DNA molecules. These nitrogen containing DNA molecules may interact with other bacteria.

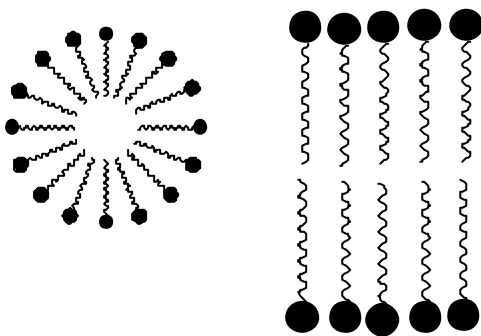
In figure 4 the schematics of a bacterial cell are shown.

Figure 4. Bacterial structure; cell membrane, plasmids and vacuoles in water.



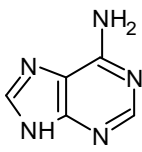
The cell membrane can be surrounded by a cell wall if a bacterium has to survive, for example under extremely dry conditions, they may produce "spores". The cell membrane consists of a lipid double layer (protein) and acts as a kind of micel in water as can be seen from figure 5.

Figure 5. Micelles as part of the bacterial cell membrane.

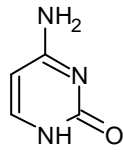


The plasmids are pieces of DNA circles and are constructed from 4 nitrogen containing bases and deoxyribose (see the chemical formulas below).

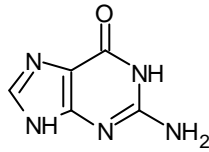
See also the similarity with nucleic acids in section: nucleic acids.



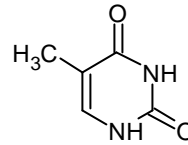
Adenine



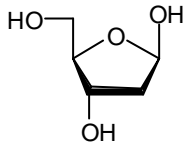
Cytosine



Guanine



Thymine



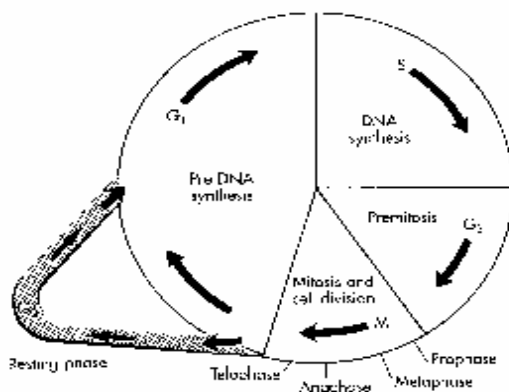
Deoxyribose

Vacuoles are closed spaces where bacteria deposit trash and store pigments. Other constituents of the bacterial cell like ribosomes play an important role in the protein synthesis. Bacteria also contain DNA structures and need the element of nitrogen to grow.

In figure 6 the schematic of a reproducing micro-organism is shown.

Figure 6. Growth cycle for mammalian cells. (lit. 10).

In the G₀ (resting phase), the cells are dormant. A variety of stimulants, often of unknown origin, cause cycling of cells to begin by entry into the G₁ phase (pre-DNA synthesis). Here precursors for DNA are formed. In the S, or synthetic phase, DNA synthesis occurs. This is followed by premitotic synthesis and structural developments in the G₂ phase. Mitosis occurs in the M phase to produce two cells, each of which can continue to cycle, by entry of again into G₁, or can enter the resting phase G₀. Growth fraction is defined as the total cells in the growth cycle (G₁, S, G₂, M) divided by the total cells (G₁, S, G₂, M, G₀).



Basically there are four groups of bacteria:

- coccen (spherical shape)
- bacilles (stick shape)
- comma bacteria (bended stift shape)
- spirilles (screw shape)

Another classification of bacteria is based on the fact, wether or not they consume inorganic oxygen. Aerobic species need the oxygen molecule for electron tranfers, while anaerobic species may find other compounds (no oxygen) for their redox-reduction processes.

The sulphate reducing bacteria (*Desulfobacter*, *Desulfonema*, *Desulfobulbus*) are examples of the anaerobic species, which are feared for their possible corrosion attack of piping and sewers (lit. 2; production of H₂S).

Bacteria remedy.

In principle there are two ways to prevent bacterial growth: physical or chemical remedy. Physical procedures are applied by radiation, e.g. radioactivity, UV light or microwaves, but also heat and steam can be used to sterilise a product.

The chemical approach is applied by addition of so called antibiotics (lit 5).

Antibiotics may be classified, based on their way of attack:

1. Cell membrane synthesis inhibitors; (*penicillin, cephalosporin*)
2. Protein synthesis inhibitors; (*aminoglycoside, chloramphenicol, tetracyclin*)
3. Folium synthesis inhibitors; (*sulfonamide*)
4. DNA transcription inhibitors; (*chinolone, nitrofurantoin*)
5. Damagers of the cell membrane; (*polymyxin*)
6. Methenamine; (*generator of formaldehyde*)

The above mentioned physical and chemical bacteria killers are all acting in such a way that essential processes in the organism are disturbed or part of it is completely destroyed.

A third way to prevent bacterial growth can be organised by creating an environment in which (bacterial) life is impossible e.g. high or very low temperatures. In this case the absence of water and nitrogen is proposed to assure that no bacterial growth is possible.

Bacteria need energy (food) to continue their life cycle.

Separately from the redox-processes the bacteria also need carbon and nitrogen for their reproduction. Carbon can be supplied by many organic compounds or from carbon dioxide. For example carbohydrates are perfect energy sources for bacterial growth. But for bacterial growth also other essential elements (phosphorus, sulphur and nitrogen) are required, to build new proteins for cell walls and to produce nucleotides to construct new nucleic acids.

Important nitrogen suppliers are amines and amides, ammonium, nitrate, nitrite but also organic nitrogen compounds as well as elemental nitrogen may feed their reproduction process. Moist (the presence of water) is another requirement for bacteria to reproduce.

In the context of this research on the lack of nitrogen in the protective coating Stopaq wrapping band, this issue is studied in a literature search (see chapter 5).

5. Literature search

In order to verify the hypothesis mentioned in section 4 an additional literature search in scientific literature is performed. The intention of this search is to find publications which describe the lack of nitrogen in microbiological (anaerobic) cultures.

The question is if no nitrogen is available what will be the reaction of the culture.

It is known that micro-organisms can also convert atmospheric nitrogen gas for their need of surviving or to produce nitrate or nitrite as an intermediate for other cultures.

In such a symbiosis a mix of microbiological cultures may produce ammonium as a the feed for other anaerobic cultures.

(Acknowledge is appreciated for the literature search; applied by scientific employees of the University Groningen; drs. U. Kooystra and dr. S. Kuindersma)

The target of the literature search is to find scientific papers which describe conditions of nitrogen lack in bacteriological cultures.

Since there are bacteria who can also consume inorganic nitrogen (N₂ from air), these publications are also studied.

*The key words to find these references in Biological Abstracts (1990 - 2003) are:
bacteria; absence of nitrogen; lack of nitrogen; deprivation of nitrogen, without (free) nitrogen.*

Results:

The literature search results in a hit of 173 publications concerning the influence of nitrogen lack in bacterial media.

In an additional document to this report (Appendixes) these abstracts are shown.

None of these papers are describing the absolute absence of nitrogen in biological cultures.

This can be explained as that all organisms need nitrogen for reproduction.

It means that without nitrogen there is no bacterial growth.

Most of the studies handle the concentration of nitrogen and the type of the nitrogen source (e.g. NO₃⁻, NO₂⁻, NH₄⁺).

For example:

record 2

the cyanobacterium (*Spirulina platensis*) was investigated on inorganic nitrogen concentrations (ammonium, nitrite, etc.) for shrimp growth. The scientists found competition between the shrimp growth and the presence of the bacteria (record 2).

record 5

different nitrogen sources are used to study the survival of *Corynebacterium glutamicum* at nitrogen limitation. They find several mechanisms of the bacteria to adapt their metabolism to changes in the nitrogen supply. Still the micro organisms need nitrogen.

record 6

nitrogen deprivation led to arrested cell growth and increased cellular C:N ratios.

record 24

addition of organic carbon and nitrogen is necessary for sulfate reducing bacteria.

record 32

cells acclimate to nitrogen deprivation by differentiating into non-pigmented resting cells, which are able to survive prolonged periods of starvation.

record 51

bacteria grow on amino acid containing media and are unable to consume ammoniumchloride; this leads to inhibited growth.

record 54

biochemical measurements revealed an increase of oxygen consumption during nitrogen starvation, indicating an enhanced energy demand of the cells.

record 70

nitrogen starvation indicates extensive differential gene expression.

record 71

factors determining cyanobacterial success in ecosystems; cyanobacteria have low competitive ability for nitrate compared with algae and high ability to compete for ammonium.

record 72

nitrogen isotope study; two functional groups of bacteria in soil, one uses protein for energy and C, N source, the other uses carbohydrate as an energy and carbon source, while ammonium is the nitrogen source, obtained after mineralisation by the first group of bacteria.

record 80

Methylobacterium thiocyanatum is able to grow on thiocyanate or cyanate as the only nitrogen source.

record 104

changes in nitrogen source modify distribution of excitation energy in cyanobacterium.

record 129

aniline and nitrobenzene supported growth in an N₂ atmosphere, during the absence of other nitrogen sources in the medium.

record 142

nitro aromatic compounds serve as nitrogen source for sulfate reducing bacteria (sole dinitro toluene is the source of growth and electron acceptor).

record 164

cyanobacterium anabaena responds to nitrogen deprivation by forming of nitrogen-fixing cells (some eubacteria have the ability to fix free nitrogen from the air and to convert it to nitrate).

record 165

bacterial strain grows solely on carbon source (Tween or propionate) and nitrogen fixing ability.

record 169

cyanobacterium anabaena bacteria are known to fix nitrogen when deprived of combined nitrogen sources under anaerobic conditions; if no nitrogen at all is available morphological distinct cells appear, like heterocysts (food storage and rest of reproductive structure), while the filaments are starving and fragmenting.

record 170

betaine (trimethylammonium compound) cannot be used as a nitrogen source, not even in a situation of total nitrogen depletion for the bacterium of *Ectothiorhodospira halochloris*.

Bacteria are able to mutate, during deprivation, in order to find new elemental sources of carbon and nitrogen. Some species are able to convert nitrogen from air to produce nitrates. Bacteria are capable to survive for a long period of time and they can adapt under different conditions or live in symbiosis with other cultures, but anyway they need nitrogen for growth. The evaluation of this literature search leads to the conclusion that nitrogen is required for the growth of micro organisms.

6. Experimental conditions

In the first phase of this research project two coating samples are analysed. One new product (R) and a sample (A) of a used protective coating Stopaq wrapping band is analysed by microscopy (SEM/EDS). Additionally a qualitative test for the forming of magnetite is performed, because this iron crystal was assumed to form, due to the application of cathodic protection. The used wrapping band sample has been taken from an original metal transport line which was already in use.

Next the surface of the protective coating is extracted with THF (tetrahydrofuran) to examine the solution with GC/MS (gaschromatography with mass spectrometric detection).

Also FTIR (Fourier Transform Infra Red) spectra are recorded from both samples.

Instrumental parameters.

SEM/EDS

By scanning electron microscopy/ energy dispersive spectrometry (SEM/EDS) a detailed view of the material surface is obtained. At the same time this technique produces a global semi-quantitative analysis of the composition of the material.

pH measurement of the adhesive surface material.

A wet pH indicator test stroke is put on the top of the adhesive side of the coating.

Extraction and GC/MS analysis. .

Warm THF is used to wash and extract the coating surface material.

The extract is analysed by GC/MS:

Gaschromatograph	:	Hewlett Packard 5890.
Column	:	Fused Silica WCOT HP-5 (25 m x i.d. 0,25 mm; $d_f = 0,25 \mu\text{m}$)
Oventemperature	:	40 °C (4 min.) → 15 °C/min. → 300 °C
Injection	:	splitless; 275 °C.
Injection volume	:	1 μl
Detector	:	Hewlett Packard 5792 series Mass Selective Detector; 165 °C. m/e 1,2-700
Software library	:	Sadtler library with mass spectra.

FTIR/ATR.

Attenuated Total Reflection (ATR) with a single reflection diamond probe is used as the sampling unit. Spectra are recorded from 4000 - 600 cm⁻¹.

FTIR spectrometer: Perkin Elmer system 2000

Sampling technique: ATR - Golden Gate with diamond crystal.

In the second phase the literature search is performed (see chapter 5)

In the third phase of this research the ingredients of the formula of the Stopaq wrapping bands are analysed for nitrogen containing components.

Normally nitrogen analysis by the Kjeldahl method is a standard technique to use.

However, the complete coating product is very difficult to destruct with sulfuric acid.

So another very sensitive nitrogen detection test is used to screen all the ingredients and the final product on the presence of nitrogen. All nitrogen containing components in the sample will be converted to nitrogen monoxide (NO), which latter component is detected.

The semi-quantitative Griess method is evaluated and briefly validated (appendix A).

In the Griess test a sample is mixed with manganese dioxide in a test tube and strongly heated with a microflame. The test tube is covered with a disc of filter paper moistened with the Griess reagent. A positive response is indicated by the development of a pink or red circle on the colorless paper.

The validation results are shown in appendix A. From these data it can be seen that the detection limit for nitrogen nutrients is < 10 ppm elemental nitrogen (w/w) in the test sample.

Next the Griess nitrogen test is applied to all ingredients and final products of the Stopaq wrapping bands.

Microbiological tests.

Additionally some samples of the Stopaq products are tested for their bacterial activity after production. The results may show whether or not the original material is sterile.

Three tests cultures are applied to detect the initial bacterial activity of the Stopaq product:

- colony forming units (CFU); Hygicult-TPC
- Enterobacteriaceae (ground bacteria) and glucuronidase;
Hygicult E/ β -glucuronidase (β -GUR)
- yeast and fungi; Hygicult Y & F

The test material is manufactured by Orion Diagnostica, PO Box 83, Espoo, Finland.

Received samples:

On 17-6-2003 samples of the wrapping bands were received by CS Aspa Laboratories at Gieten.

Code:

- A. wrapping band CZ H (sample of used product)
- R. reference sample CZ H

- C wrapping band CZ H (new)
- D wrapping band CZ (new)

Also samples of all ingredients of the Stopaq wrapping band formula are separately obtained: 19 products dd. 20-6-2003.
(see table V, under results nitrogen test).

7. Results and Discussion

SEM/EDS; microscopic view.

The chemical composition of sample (A) en reference (R) is determined by EDS. From the results only the expected elements like carbon, oxygen and calcium, minorities of magnesium, aluminium en silicium are detected (see table IV and Appendix B). This means that the major material is built from organic components and a limestone filler. No iron is detected on the used wrapping band (A), which means that no magnetite could be measured.

Tabel IV: Global composition of the CZH wrapping band surface.

Element	sample R % (w/w)	sample A % (w/w)
carbon; C	39	38
oxygen; O	27	29
calcium; Ca	32	33

See also Appendix B

Measurement of pH.

Pressing a wet pH paper to the adhesive surface resulted in a pH value of 8,0 for both samples (A and R). This slightly basic condition is due to the carbonate in the sample.

Extraction and GC/MS analysis.

The warm THF is able to dissolve a thin layer of the sample material. The extract also contains insoluble small particles of the filling material of the coating. After filtration and concentration (partly evaporation) the THF solution is analysed by GC/MS. The chromatograms show 4 peaks of hydrocarbons and carboxylic acid as part of the ingredients of the coating product. No nitrogen containing components are detected. The chromatograms are shown in Appendix C, containing: (tetradecane, stearic acid, butylhydroxytoluene and a phtalic acid ester).

FTIR/ATR

A small amount of the sample is used for the recording of the IR spectrum.

The spectra show some distinct peaks of chemical functional groups:

CH₃ and CH₂ stretch vibrations at 2950 cm⁻¹ en 2890 cm⁻¹; confirmation of hydrocarbon and similar with the results of the GC/MS analysis.

From the other specific peaks no special information can be obtained.

It is clear that no nitrogen functional groups are recognised in the spectra;
(no amines: 3200-3500 cm⁻¹, nor amides: 3050-3350 cm⁻¹).

The spectra are shown in Appendix D.

Nitrogen availability test

The Griess nitrogen test is applied for all ingredients and final products of the wrapping band. In table V the results are summarized.

From these data it can be seen that the detection limit for nitrogen is < 10 ppm N (w/w) in the test sample.

Table V. Nitrogen test results of wrapping bands and ingredients.

- = negative on total nitrogen (< 10 ppm w/w nitrogen)

+ = positive; (nitrogen detected)

<i>Sample Code</i>	<i>Description</i>	<i>Result</i>		
		<i>Test I</i>	<i>Test II</i>	<i>Test III</i>
03061301	ingredient 1	-	-	-
03061302	ingredient 2	-	-	-
03061303	ingredient 3	-	-	-
03061304	ingredient 4	-	-	-
03061305	ingredient 5	-	-	-
03061306	ingredient 6	-	-	-
03061307	ingredient 7	-	-	-
03061308	ingredient 8	-	-	-
03061309	ingredient 9	-	-	-
03061310	ingredient 10	-	-	-
03061311	ingredient 11	-	-	-
03061312	ingredient 12	-	-	-
03061313	ingredient 13	+	-	-
03061314	ingredient 14	-	-	-
03061315	ingredient 15	-	-	-
03061316	ingredient 16	-	-	-
03061317	ingredient 17	-	-	-
03061318	ingredient 18	-	-	-
03061319	ingredient 19	-	-	-
03061320	sample C: wrapping band CZ H	-	-	-
03061321	sample D: wrapping band CZ	-	-	-
--	polyethylene film (LDPE)	-	-	-
--	polyethylene carrier (HDPE)	-	-	-

There is no relation between the number of ingredients and the formula of the wrapping bands. More similar ingredients of different manufacturers have been tested.

All ingredients and the products of the wrapping band itself are negative for the nitrogen test. This means that the coating is a poor medium for micro organisms to grow (< 10 ppm N).

Microbiological tests.

The production of the Stopaq wrapping band is performed during temperatures of at least 110 °C, which means that the production process is rather sterile.

The next detection tests are applied:

- a.) CFU (colony forming units): general test for many bacteria
- b.) Enterobacteriaceae (anaerobic bacteria)
- c.) Yeast and fungi

(test set data are described under Experimental; chapter 6)

The development of microbial activity is measured after 24 and 72 hours of incubation after inoculation. The incubation temperature for each test is 37 °C except for the yeast and fungi test which is 24 °C. The microbe activity is expressed as a general accepted value of colony forming units per square cm (CFU/cm².)

To validate these test also a blanc and an on purpose infected Standard is measured.

In table VI the microbiological tests are summarized:

Table VI. Bacteriological tests of the wrapping band products.

Specific test	Sample	Activity after 24 hr. <i>CFU/cm²</i>	Activity after 72 hr. <i>CFU/cm²</i>	Total result
Total bacteria				
	R (ref.)	none	none	sterile
	C	none	none	sterile
	blank	none	none	sterile
	Standard	5	--	infected
Bacteriaceae				
	R (ref)	none	none	sterile
	C	none	none	sterile
	blank	none	none	sterile
	Standard	50	> 100	infected
β-Glucuronidase				
	R (ref)	none	none	hygienic
	C	none	none	hygienic
	blank	none	none	hygienic
	Standard	> 50	>100	non-hygienic
Yeast and Fungi				
	R (ref)	none	none	sterile
	C	none	none	sterile
	blank	none	none	sterile
	Standard	0	3	fungi

R = reference sample CZ H; C= wrapping band CZ H (new)

Blank = test with same procedure, without sample

Standard = test with infected sample (to validate the proper working of the test)

8. Evaluation of results.

Hypothesis of "No nitrogen, No bacterial growth".

It is clear from theoretical approach and from literature research that nitrogen is absolutely required for bacterial multiplication. The nitrogen element is an essential brick in the construction of new proteins and the genetic DNA molecule.

A bacterial cell contains approximately 10 % dry material, which dry material contains 12 % nitrogen (elemental). Hence, if no nitrogen is available, bacterial growth is impossible.

Detection of nitrogen

The ingredients of the Stopaq wrapping band are tested on their total nitrogen content.

No nitrogen or nitrogenic nutrients in the ingredients and in the final product of the wrapping band are detected (detection limit < 10 ppm).

This means that bacterial growth is very limited.

Bacterial growth and water requirement

All chemical processes within a bacterial cell are performed in aqueous environment.

This means that water is essential for bacterial life.

Permeation of water (high polarity) through the wrapping band is practically impossible, because the composition of the apolar wrapping band is water repellent (phase separation and no swelling of salt crystals from water diffusion; see chapter 2, page 7).

For bacterial growth additional water, carbon and nitrogen is required to produce new cells.

Since no water is available, bacterial growth is improbable.

Permeation of water and Nitrogen

Because bacteria are capable to consume elemental nitrogen, they may feed themselves from nitrogen from the environment. Since the transport tubes are buried in soil there is hardly any air and elemental nitrogen available. It is possible that groundwater saturated with nitrogen is in contact with the outside wall of coating systems.

Generally speaking, all organic and polymer coating products are permeable for water and nitrogen. Water and nitrogen may diffuse only through the protective layer if a driving force is present; e.g. in the form of a consuming bacterium at the surface of the metal tube.

Since there is only a limited amount of gas available in the surrounding area (soil) the total feed is very limited [*solubility of nitrogen in water: 300 ppm (w/w); diffusion coeff. of nitrogen in water: 2.35×10^{-9} m²/s. ;(lit. 13, 14)*].

The permeability of water, oxygen and nitrogen through the 3-layer wrapping band has been studied and is very low according to the results of former reports (lit.6). Since the piping with protective coating is buried in soil only a limited amount of nitrogen is present in the outside environment.

Conventional coatings are mostly hardening in time, which make them fragile and porous, creating spaces and fractures in the coating layer.

However, the fluid Stopaq wrapping band will fill up the micro pores at the metal surface by its permanent adhesion property. No fragile parts (cracking) will develop in the permanent viscous-elastic material (no driving forces for permeation).

For reproduction, micro organisms require nutrients and a favourable environment. So it is important to evaluate the environmental circumstances and the specifications of the wrapping band for microbial attack.

The main precautions and conditions which are negative for bacterial growth are summarized:

- no water is present at the boundary of metal/ wrapping band
- no nitrogen available in the wrapping band
- no initial bacterial activity is present at the wrapping band (sterile)
- permanent tightly closed wrapping band system due to strong adhesion at the interface (only cohesive fractures)
- wrapping band system is under permanent over pressure (buried in soil)
- self recovering behaviour of Stopaq due to its permanent viscous-elastic (fluid) character
- very low permeability of the 3-layer wrapping band (PVC, PE and Stopaq) for water and nitrogen
- limited amount of free elemental nitrogen in soil and water
- no permeability for ionic species from soil, e.g. nitrate, nitrite, ammonium (no water is available and the ions are insoluble in the apolar Stopaq material)
- Stopaq is water repellent due to its apolar ingredients
- Stopaq is slightly basic (pH=8); unfavorable for SRB

9. Conclusion

The permanent fluid Stopaq wrapping band consists of an organic polymeric composition with inorganic filler material. It is proposed that if no nitrogen nutrients are available in the coating substance, it is impossible for microorganisms to grow on this material under anaerobic conditions.

From the literature study and the theoretical principles explained in this report the hypothesis "no nitrogen, no bacterial growth" is true.

The ingredients of the wrapping band formulas and the coating itself are tested on their total nitrogen content and result in the absence of any nitrogenic nutrient (< 10 ppm N w/w).

Other analytical techniques (GC/MS and FTIR) confirm these results that no nitrogen could be detected.

The Stopaq wrapping band is water repellent because of its hydrophobic property and permeation of water through the 3-layer wrapping band can be neglected. It is clear that if no water is present, bacterial life is impossible.

For the Stopaq wrapping band application a large life span may be noticed, because microbiologically induced corrosion (MIC) of metal transport tubes is improbable. The conditions for the growth of micro-organisms are very unfavourable:

- no water is available
- no nitrogen is available for bacterial growth
- the wrapping band is produced under sterilising condition (high temperature)
- the basic conditions (pH=8) of wrapping band prevents the development of SRB
- the self-recovering ability of the permanent viscous-elastic fluid material enhances the protection properties (*no cracks during long period of time*)

Empirically results of two field tests during a period of 7 years confirm that during this period of time no bacterial corrosion was observed.

Finally, it may be concluded that microbial growth at the interface of the metal tube and the wrapping band is practically impossible.

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