Dehalogenimonas: un nuovo gruppo di batteri capace di degradare composti clorurati come PCE e TCE ad etene

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SOMMARIO

Gli idrocarburi clorurati sono ampiamente utilizzati nell'industria come intermedi chimici o solventi. A causa del loro stoccaggio improprio e di fuoriuscite, questi composti si trovano frequentemente nell'ambiente, nel quale sono tossici e persistenti. La biodegradazione degli idrocarburi clorurati è un processo importante e spesso utilizzato per la bonifica di siti contaminati. Fino a poco tempo fa, si pensava che la degradazione completa degli eteni clorurati (PCE e TCE) in etene fosse a carico esclusivamente di organismi di un solo genere: *Dehalococcoides*. Recentemente è stato scoperto che anche i batteri del genere *Dehalogenimonas* sono capaci di declorazione completa in etene. Nel nostro studio, presentiamo i risultati di analisi molecolari del DNA da 14 pozzi di monitoraggio da un sito nella città 's Hertogenbosch nell'Olanda meridionale, che mostrano un alto numero di batteri del genere *Dehalogenimonas* vicino a uno strato di TCE in prodotto puro, indicando sostanziale degradazione naturale a carico di questi batteri. Con l'aiuto di nuove tecniche di analisi del DNA (Next Generation Sequencing e PCR quantitativa) presso il laboratorio di Orvion, è stato possibile identificare la popolazione microbica a monte, vicino alla zona del prodotto puro e a valle del sito. Fino all'80% della popolazione batterica vicino alla zona di origine del TCE è costituita da batteri del genere *Dehalogenimonas*, mentre il genere *Dehalococcoides* non risulta particolarmente dominante. L'analisi qPCR ha misurato concentrazioni di batteri *Dehalogenimonas* vicine a 5 milioni di cellule ml-1, paragonabili a colture attive commerciali. Inoltre, in uno dei pozzi di monitoraggio analizzati, è stato rilevato il gene *cerA*. Questo gene codifica per un enzima coinvolto nelle fasi finali della completa degradazione in etene, ed è l'unico conosciuto finora dal genere *Dehalogenimonas*. I risultati di questo studio sono importanti per l'applicazione di tecniche di biorisanamento, poiché indicano altre possibilità oltre a *Dehalococcoides*. Infatti, anche il genere *Dehalogenimonas* dovrebbe essere analizzato quando si cercano prove della presenza di attenuazione naturale dei composti clorurati in siti contaminati.

Parole chiave: biorisanamento, analisi molecolari, Dehalogenimonas, Dehalococcoides, siti contaminati

ABSTRACT

Chlorinated hydrocarbons are widely used as in the industry as chemical intermediates or solvents. Due to improper storage and spills, these compounds can be found frequently in the environment, in which they are toxic and persistent. Biodegradation of chlorinated hydrocarbons is an important process and often used for the active remediation of contaminated sites. Until recently, complete degradation of chlorinated ethenes (PCE and TCE) to harmless ethene was thought to be performed exclusively by organisms of only one genus: *Dehalococcoides*. Recently it was found that bacteria of the genus *Dehalogenimonas* are also capable of complete dechlorination to ethene. In our study, we present DNA results from 14 monitoring wells from a site in the city 's Hertogenbosch in Southern Holland, that show high numbers of *Dehalogenimonas* near a pure-product TCE layer, indicating substantial natural degradation by this species. With the help of novel DNA techniques (Next Generation Sequencing and quantitative PCR) at the laboratory of Orvion, it was possible to screen and identify the microbial population upstream, near the pure product zone and downstream at the site. Up to 80% of the bacterial population near the TCE source zone consists of bacteria of the genus *Dehalogenimonas,* while genus *Dehalococcoides* is not highly enriched. The qPCR analysis measured concentrations of *Dehalogenimonas* bacteria close to 5 million cells ml^{-1}, comparable to commercial active cultures. Additionally, in one of the monitoring wells analyzed, gene *cerA* was detected. This gene is involved in the final steps of the complete degradation to ethene, and it is the only one known so far from genus *Dehalogenimonas*. The findings of this study are important for bioremediation applications, as they point towards more possibilities other than *Dehalococcoides*. In fact, *Dehalogenimonas* should also be analyzed when searching for proof of natural attenuation of chlorinated compounds in contaminated sites.

Key words: bioremediation, molecular analysis, Dehalogenimonas, Dehalococcoides, contaminated sites

1. INTRODUCTION

Chlorinated hydrocarbons are widely used as solvents and raw materials for the synthesis of various useful products, such as cleaning agents, pesticides and poly vinyl chloride (PVC). However, there is concern regarding the toxicity and carcinogenic properties of some of these compounds. They are widely present in the environment because of incidental spills and improper disposal. These compounds can easily reach the groundwater and drinking water aquifers, and they can reach concentrations close to their solubilities, forming pure product zones. Additionally, they tend to accumulate at the bottom of the water table, forming DNAPL (dense nonaqueous phase liquids) layers. These can slowly dissolve in flowing groundwater, acting as continuous source of release of contaminants (Fogel, M. M. et al., 1986).

Chlorinated ethenes such as trichloroethylene (TCE) and perchloroethylene (PCE) are mainly used for dry cleaning, metal degreasing, as chemical intermediates and as solvents. They are persistent in the environment and their degradation is very limited under aerobic conditions (Czinnerová, M. et al, 2020). Biological degradation is better achieved under anoxic conditions and until recently, bacteria of the genus Dehalococcoides were believed to be the only ones able to perform complete degradation of these compounds to non-toxic ethene. Studies in the early '90s showed that an anaerobic PCE-dechlorinating enrichment culture converted high concentrations of PCE to ethene at unprecedented rates and that hydrogen was used as electron donor for dechlorination. Other species (*Dehalobacter, Desulfuromonas, Desulfitobacterium, Geobacter*) were known to be able to degrade chlorinated compounds to less chloride-rich ones, such as dichloroethylene (cis-DCE) and vinyl chloride (VC). The latter is especially toxic and suspected carcinogenic, thus its removal is desirable in a bioremediation approach (Löffler, F. E. et al., 2013). Only recently, in 2017, one study showed that Dehalogenimonas species were also able of complete dehalogenation of PCE and TCE to ethene (Yang, Y. et al., 2017).

In our study we used a combination of Next Generation Sequencing (NGS) and qPCR techniques to investigate the bacterial population of a site in the city 's Hertogenbosch, the Netherlands. The site was previously a production site and is contaminated by particularly high concentrations of TCE, cis-DCE and VC. In total 14 monitoring wells were sampled and analysed by qPCR for selected bacterial targets. On 4 of them, a NGS analysis was also performed to gain insight in the whole bacterial population and dominant species. The aim of the project was to quantify known degraders in the samples and identify the dominant species present in the contaminated wells and their correlation with the degradation of the contaminants of interest. Based on this knowledge, it would be possible to assess the potential for natural attenuation and suggest the best options for stimulating the degradation of the contaminants to be applied in a bioremediation plan. In our analysis, we found *Dehalogenimonas* to be dominant and present at higher concentrations than *Dehalococcoides* in the source zone of the site.

2. MATERIALS AND METHODS

2.1 Samples collection and processing

The wells were sampled following the Dutch guidelines for groundwater sampling. Samples were selected in the source (high concentrations of TCE in particular) at various depths and in the plume. Clean reference monitoring wells have been selected as well, at three depths. The samples were filtered with a 0,2 μm cellulose filter (Carl Roth) and the DNA was isolated using the PowerFecal Pro kit (Qiagen).

2.2 Molecular analyses - qPCR

On all 14 samples qPCR analysis were performed on the following targets: *Dehalococcoides* spp., *Geobacter* spp., *Dehalogenimonas* spp., *etnC* and *cerA* (VC degradation genes). The procedures used have been developed by Orvion and are confidential. More information can be found at the link in the references list ("Quality of ORVIDetect analyses").

Figure 1. identification of the three water flow paths under analysis at the site

Flow path 1:					
PB23 (4-10) Reference	4 (3.5-4.5)	PB3 (4-10)	PB4 (11-17)	PB4 (18-24)	
Flow path 2:					
PB12 (18-24) Reference	PB01 (18-23)	PB4 (18-24)			
Flow path 3 (aquifer):					
PB41 (30-50) Reference	PB27 (29-39)	PB28 (29-39)	PB34 (29-38)	PB40 (30-50)	PB36 (32-52)

2.3 Molecular analyses – NGS

On 4 samples, a NGS analysis was performed using the Oxford Nanopore Technology. More information can be found on their website, with reference to the library prep kits. The raw data has been analysed using a software developed by Orvion.

The DNA reads produced by the NGS analysis were compared to a custom-made nucleotide database and the bacterial population in the samples was identified up to genus level. The results were visualised in graphs, available also as interactive files.

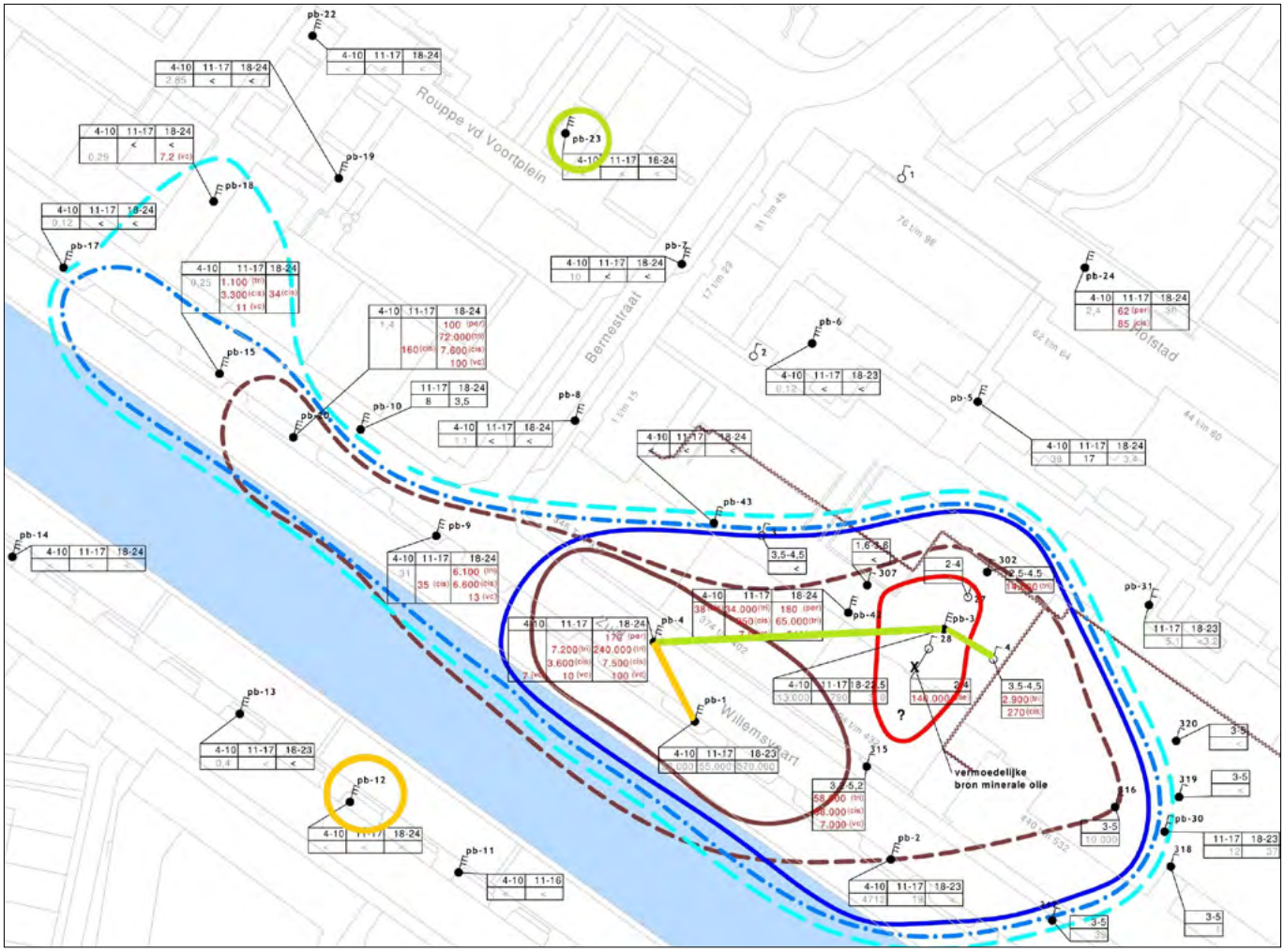

FIGURE 2. Flow path 1 (green) and 2 (yellow) with the references (circled in green and yellow).

3. RESULTS AND DISCUSSION

3.1 Water flow path analysis

Three water flow paths were identified at the site and are their composition is shown in the scheme below (Figure 1).

The first flow path runs from shallow (3.5-4.5 m-sl) to deeper (18-24 m-sl). The high concentration of TCE is located on a less permeable layer (loam/clay) that is present at approximately 24 m-sl. Due to the high concentration of TCE (140,000µg/l) there may be a layer of pure product. Even higher concentrations have been measured in the past, which makes the presence of pure product TCE very likely. However, the presence of pure product has not been conclusively established. The second flow path runs just over the loam layer at 24 m-sl, from monitoring well 1 to the source in monitoring well 4. The third flow path is located in the first aquifer, which starts at approximately 28 m-sl. There is leaching of contamination from the separating layer, as there is a long plume in the first aquifer. The flow path starts in monitoring well 27 and runs to the tip of the plume (monitoring well 36), which is situated about 500 m downgradient. The flow paths are represented in Figures 2 and 3 below.

3.2 qPCR results

The results of the qPCR analyses are presented in Appendix. Visually, the results are shown in Figure 4.

3.2.1 Dehalococcoides sp.

Bacteria of the genus *Dehalococcoides* were not found in the references, with the exception of a low number in monitoring well 12 (18-24 m-sl). This shows that *Dehalococcoides* does not or hardly occur naturally in the groundwater. These bacteria are able to completely reductively dechlorinate PCE and TCE to the harmless ethylene/ethane. The results show that the numbers in flow paths 1 and 2 are only slightly increased, on the order of 100 to 1.000 cells ml^{-1}. In the first aquifer (flow path 3), this species was not present in the reference, but was clearly elevated in the plume up to monitoring well 40, where about 20.000 cells ml^{-1} were found. In the most downstream monitoring well 36, *Dehalococcoides* was however not found (< 44 cells ml^{-1}). Natural attenuation thus occurs in the plume of the first aquifer up to monitoring well 40. No or considerably less natural attenuation by *Dehalococcoides* occurs in the tip of the plume.

3.2.2 Geobacter

Bacteria of the genus *Geobacter* are widespread in the soil. *Geobacter* bacteria are able to effectively break down PCE and TCE into cis-DCE. Further degradation to VC and ethylene/ethane is not known from *Geobacter*. Because *Geobacter* species are motile, they grow towards high concentration zones of PCE and/ or TCE. Geobacter was detected in all three references. Higher numbers were found in the heavily polluted monitoring wells (PB 4, 11-17 m-sl

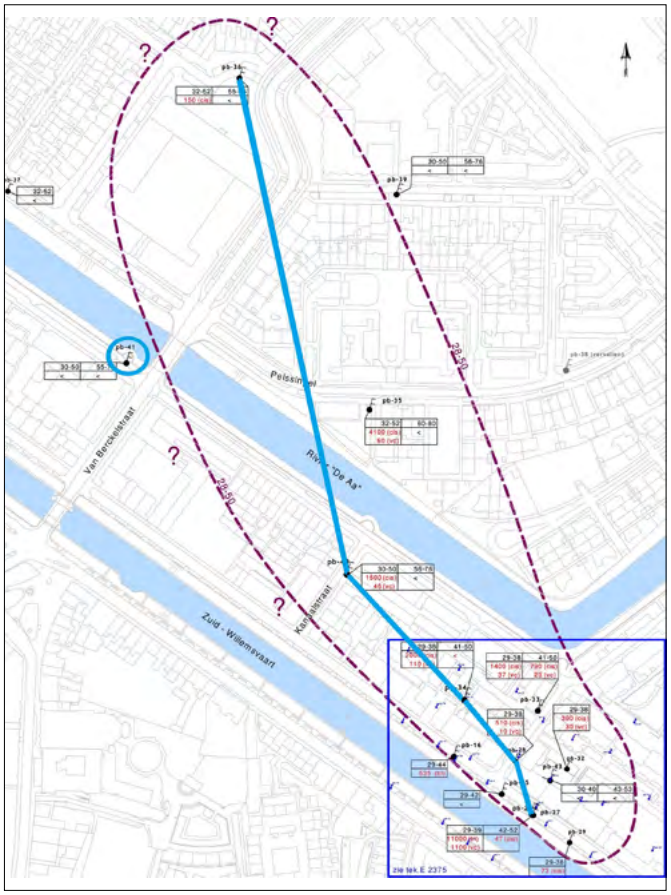

FIGURE 3. Results of the NGS analyses (relative abundance, %, left axis) related to dehalogenation and concentrations of the contaminants (µg/l, right axis). Bars colours: *Dehalogenimonas* (blue), *Geobacter* (yellow), *Dehalococcoides* (orange). Line colour: TCE (orange)

and 18-24 m-sl). *Geobacter* was present, up to 98.000 cells ml^{-1}, in the deepest and most contaminated filter (PB4, 18-24 m-sl). This means that *Geobacter* actively contributes to the conversion of TCE to cis-DCE in the source zone. In the first aquifer, *Geobacter* does not have a significant role in the degradation of the chlorinated ethenes, as the numbers are not increased compared to the reference.

3.2.3 Dehalogenimonas and cerA

In the core, high numbers of Dehalogenimonas were found (4.600.000 cells ml^{-1}). Also, in the plume (first aquifer), the numbers have clearly increased compared to the reference and quite high numbers were found up to the tip of the plume (monitoring well 36, 100.000 cells ml^{-1}). These results indicate that, particularly in the source but also in the plume, degradation of chlorinated ethenes by Dehalogenimonas takes place. Whether the degradation occurs everywhere and whether it is complete to ethene/ethane is not certain. To gain more certainty about this, additional analyses were performed on DNA encoding the cerA gene. This gene is specific for Dehalogenimonas and is used to convert vinyl chloride to ethene. The cerA gene was only found in monitoring well 34 (29-38 m-sl) in the first aquifer. In this spot it is then sure that Dehalogenimonas breaks down the chlorinated ethenes to ethene/ethane. However, this can still also be the case in other places, due to the fact that knowledge of the cerA gene is still very limited and the genetic basis for designing the qPCR assay is still very narrow. This means that it is quite possible that the current analysis cannot detect all cerA DNA sequences at the moment. Given the high concentrations of Dehalogenimonas, in the source contamination (pure product TCE) there is a significant biological process happening that we have not encountered before under natural conditions at this intensity. Dehalogenimonas plays an important role in natural breakdown of chlorinated ethenes also in the plume, based on the results of the qPCR analysis.

3.2.4 etnC

Analyses were also performed on DNA encoding the etnC gene. This gene is used by various bacteria for aerobic/microaerophilic degradation of cis-DCE and VC. This requires oxygen and the compounds are broken down into chloride and CO_2. The gene is present in shallow flow path 1 and is most commonly found in shallow monitoring well 4 (3.5-4.5 m-sl). This has to do with the availability of oxygen, which is greater in shallow layers. Thus, some aerobic degradation of cis-DCE and VC also occurs in the shallow soil layers. This process is not present in the deeper soil and in the first aquifer.

3.3 NGS results

With NGS analysis, the biodiversity in reference monitoring well 12 (18-24 m-sl), monitoring well 1 (18-23 m-sl), monitoring well 4 (18-24 m-sl) and monitoring well 40 (30-50 m-sl) was determined. The results are presented in an interactive file, of which a screenshot of the species related to dehalogenation can be found in figure 3 below.

The results show that in the source (PB4, 18-24 m-sl) 83% of the bacterial population consists of *Dehalogenimonas*. Also, in monitoring wells 1 and 40, the dominance of *Dehalogenimonas* is significant (50% and 16%, respectively). An undefined bacterium was also found in monitoring wells 1, 4 and 40; because it does not appear in the reference, this could be a yet unknown species involved in dechlorination processes (not shown). In monitoring well 4 in the source, more than 1% of the bacteria is of the genus *Desulfococcus*. These bacteria can break down BTEX anaerobically. Because these bacteria are only found in the core, it is conceivable that aromatics are present as secondary contamination in pure product and serve as a carbon source for the dechlorinating bacteria.

The results of the biodiversity analysis confirm the dominance of *Dehalogenimonas*, especially in the source, but to a lesser extent also in the plume. The bacterial population differs greatly from the natural population, both in source and plume. The presence of the TCE contamination leads to a strong increase of specialized dechlorinating bacteria.

4. CONCLUSIONS

The conducted research highlighted the presence in the source zone, which is heavily contaminated with TCE, of a bacterial population that is specialized in dehalogenation. Specifically, bacteria of the genus Dehalogenimonas have been found in high densities (comparable to commercially grown cultures); these bacteria play an important role in

natural degradation, both in the source and in the plume of the contamination. In the source, Geobacter also plays a role in TCE breakdown (not in the plume), while for Dehalococcoides the opposite is found, with higher densities in the plume than in the source.

This research confirms and elaborates on the findings of recent studies that identified in *Dehalogenimonas* a new dechlorinator able to completely degrade TCE, next to the already known *Dehalococcoides*. The complete degradation to ethene was found only in one of the analysed monitoring wells. Better knowledge in the genomic diversity of the gene(s) involved in this process, such as *cerA*, is needed to better investigate this subject.

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APPENDIX - QPCR RESULTS

Sample number	Sample name	Sample type	Sample number	Sample name	Sample type
001	PB12 (11-17)	Groundwater	008	PB27 (29-39)	Groundwater
002	PB12 (18-24)	Groundwater	009	PB3 (4-10)	Groundwater
003	PB34 (29-38)	Groundwater	010	PB41 (30-50)	Groundwater
004	PB28 (29-39)	Groundwater	011	4 (3,5 - 4,5)	Groundwater
005	PB01 (18-23)	Groundwater	012	PB23 (4 - 10)	Groundwater
006	PB4 (11-17)	Groundwater	013	PB40 (30-50)	Groundwater
007	PB4 (18-24)	Groundwater	014	36 (32-52)	Groundwater

qPCR target	Unit	001	002	003	004	005
Dehalococcoides spp.	copies/ml	<23	88	$3{,}2x10^3$	$3{,}2x10^3$	<20
Geobacter spp.	copies/ml	$1{,}8x10^3$	$2{,}5x10^2$	1,3x102	$1{,}1x10^2$	$7{,}1x10^2$
Dehalogenimonas spp.	copies/ml	44	24	1,9x104	$8{,}1x10^3$	$3{,}9x10^4$
etnC	copies/ml	<23	<11	<24	<25	<20
cerA	copies/ml	<23	<11	6,8x102	<25	<20

qPCR target	Unit	006	007	008	009	010
Dehalococcoides spp.	copies/ml	<105	$1{,}8x10^2$	90	$1{,}3x10^3$	<25
Geobacter spp.	copies/ml	$5{,}5x10^3$	$9{,}8x10^4$	4,9x102	$2{,}0x10^3$	$2{,}4x10^2$
Dehalogenimonas spp.	copies/ml	$8{,}7x10^4$	$4{,}6x10^6$	7,8x104	$1{,}6x10^3$	$1{,}4x10^2$
etnC	copies/ml	47	<12	<12	23	<25
cerA	copies/ml	<12	<12	<12	<12	<25

qPCR target	Unit	011	012	013	014
Dehalococcoides spp.	copies/ml	<19	<28	$2{,}0x10^4$	<44
Geobacter spp..	copies/ml	$3{,}9x10^2$	$4{,}4x10^3$	$2{,}6x10^2$	$2{,}1x10^2$
Dehalogenimonas spp.	copies/ml	64	$4{,}6x10^2$	$1{,}2x10^5$	$1{,}0x10^5$
etnC	copies/ml	$4{,}6x10^2$	<6	<16	<44
cerA	copies/ml	<12	<13	<16	<44