Acta agriculturae Slovenica, 92(november 2008)1, 75–82.

http://aas.bf.uni-lj.si

Agris category codes: P01 COBISS Code 1.01

DIFFERENCES IN MELTING TEMPERATURES OF DEGENERATED OLIGONUCLEOTIDES TARGETTING NITROUS OXIDE REDUCTASE (*NOSZ*) GENES

Blaž STRES [a)] and Boštjan MUROVEC [b)]

[a)] Univ. of Ljubljana, Biotechnical Fac., Dept. of Animal Science, Groblje 3, SI-1234 Domžale, Slovenia, Ph.D., M.Sc., e-mail: blaz.stres@bfro.uni-lj.si.

[b)] Univ. of Ljubljana, Fac. of Electrical Engineering, Tržaška 25, SI-1000 Ljubljana, Slovenia, Assist. Prof., Ph.D., M.Sc.

Received October 15, 2007, accepted February 26, 2008.
Delo je prispelo 15. oktobra 2007, sprejeto 26. februarja 2008.

ABSTRACT

One of the basic principles of molecular biology is the use oligonucleotides with comparable melting temperatures (Tm). To accommodate various evolutionary changes in target gene sequences in order to detect numerous variants of the same gene in complex microbial communities, the researchers were forced to design degenerated oligonucleotide probes and primers. In addition, recent studies suggested that relevant parameters influencing microbial activity should be included into models currently describing the final greenhouse gas emissions for public use. Further, data on microbial community structure and abundance should be included as well in near future. As one of the most potent greenhouse gases, nitrous oxide, results mainly from incomplete denitrification process, we chose nitrous oxide reductase gene (*nosZ*) as a model and surveyed published literature for *nosZ* gene oligonucleotides. We calculated *in-silico* Tm for each oligonucleotide degenerated variant and compared the resulting average Tm of both oligonucleotides used in pair. Degenerated oligonucleotides were found to contain variants differing in Tm for as much as 13 °C. More than 85% of oligonucleotides had difference in average Tm of paired oligonucleotide larger than 2 °C, more than 60% larger than 4 °C and more than 40% larger than 6 °C, 25% larger than 8 °C. By using such combinations at one annealing temperature or touch-down PCR or hybridization protocol, the full use of all degenerate variants could never be achieved thus bringing under the consideration the reaction chemistry. To increase the consistency of molecular results, a simple adjustment of Tm to at least comparable average Tm is recommended. In addition, critical evaluation of other methodological pitfalls should be regular practice in order to strengthen the value of molecular results as future public models parameters.

Key words: microbiology / molecular biology / melting temperature / oligonucleotides / *nosZ* / denitrification / models / greenhouse gases

RAZLIKE V TEMPERATURI TALJENJA ZAČETNIH OLIGONUKLEOTIDOV ZA ODKRIVANJE GENA *nosZ*

IZVLEČEK

Eden osnovnih principov molekularne biologije je uporaba oligonukleotidov s primerljivimi temperaturami taljenja (Tm). Da bi lahko z oligonukleotidi zajeli tudi evolucijske spremembe na tarčnih sekvencah istega gena znotraj kompleksnih mikrobnih združb, so se raziskovalci zatekli k uporabi degeneriranih oligonukleotidov. Nedavne študije predlagajo vključitev za mikrobe relevantnih parametrov, ki vplivajo na njihovo aktivnost, v modele, ki se trenutno uporabljajo za opis emisij toplogrednih plinov v javnosti. V bližnji prihodnosti pa se predvideva tudi vključitev

podatkov o strukturi mikrobnih združb in velikosti njihovih populacij. Ker je eden najmočnejših toplogrednih plinov, N_2O, rezultat v največji meri nepopolnega poteka denitrifikacije, sva izbrala gen za reduktazo N_2O (*nosZ*) kot model ter iz objavljene literature sestavila nabor uporabljanih oligonukleotidnih parov. Za vsako varianto degeneriranega oligonukleotida v paru sva izračunala predvideno Tm in primerjala povprečne Tm obeh oligonukleotidov v paru. Tm variant degeneriranih oligonukleotidov so se razlikovale do 13 °C. Več kot 85 % oligonukleotidov je imelo povprečno razliko Tm para > 2 °C, več kot 60 % > 4 °C in več kot 40 % oligonukleotidov je imelo Tm večjo od 6 °C. Z uporabo takih kombinacij pri eni temperaturi prileganja ali ,,PCR z zniževanjem temperature'' ali hibridizacijskih protokolih, je praktično nemogoče zagotoviti polno uporabo vseh degeneriranih variant. Našteto posledično različno vpliva na potek kemijskih reakcij prepoznave tarčnih mest. Da bi izboljšali konsistentnost molekularnih rezultatov, priporočava uskladitev povprečnih Tm para oligonukleotidov. Podobno pa je potrebno kritično oceniti druge metodološke šibke točke, da bi zagotovili uporabno vrednost rezultatov molekularnih tehnik kot bodočih parametrov v modelih.

Ključne besede: mikrobiologija / molekularna biologija / taljenje / temperatura / oligonukleotidi / *nosZ* / denitrifikacija / modeli / toplogredni plini

INTRODUCTION

Denitrification is a dissimilatory process in which oxidized nitrogen is used as an alternative electron acceptor for energy production when oxygen is limiting. As a part of the global nitrogen cycle, denitrification is believed to be responsible for the return of fixed nitrogen back to the atmosphere. Although responsible for nutrient loss in agriculture and a contribution to the greenhouse effect and the damage to the ozone layer, denitrification is also favourable in nutrient removal from wastewater and bioremediation (Tiedje, 1988). New generation of improved models describing the greenhouse gas emissions from long term ecological research field sites across Europe was initiated in 2006 (http://www.nitroeurope.eu/). At the same time, Schurgers *et al*. (2006) came up with an improved model describing anaerobiosis with water filled pore space and dynamic processes in various soils. Both studies suggested it was time to incorporate relevant parameters influencing microbial activity and on the long run, also data on microbial community structure and abundance into models currently describing the final greenhouse gas emissions.

While denitrification is considered a primarily bacterial respiratory process, it consists of four enzymatic steps carried out by nitrate, nitrite, nitric-oxide and nitrous oxide reductases. The latter is crucially involved in reduction of nitrous oxide (N_2O) to molecular dinitrogen (N_2). Generally, each enzyme is translated from mRNA transcribed from a genome or plasmid residing gene and once fully folded, contains a distribution of more conserved (active site) and more variable regions (neighbouring amino acid chains). However, complex microbial communities contain varieties of the same gene that differ slightly due to the fixation of various evolutionary events. Conserved regions of the same protein present in various bacterial lineages are preserved, but are not completely identical. This precludes the use of a single overall specific and covering oligonucleotide set that could be used in amplification or detection of all gene variants present in microbial community DNA. In this respect, researchers have come up with what appears to be an ideal solution – the use degenerate oligonucleotides to target as many different variants of the genes as possible, resulting in numerous different and degenerated oligonucleotide sets for the same gene. One of the basic principles of molecular biology is the use oligonucleotides with comparable melting temperatures (Tm) to enable their concomitant use at comparable Tm (Morris *et al*., 2002 and references herein). To verify whether published *nosZ* oligonucleotides were designed according to this principle, we calculated *in-silico* melting temperatures for all oligonucleotides, all their degenerated variants and compared the resulting average Tm of both oligonucleotides used in pair.

MATERIALS AND METHODS

Data selection

Literature on the molecular methods used for amplification of target denitrification genes from environmental samples was explored using available public databases: Medline (http://www.ncbi.nlm.nih.gov/PubMed), ScienceDirect (http://www.sciencedirect.com) and American Society for Microbiology (http://aem.asm.org/searchall). The following criteria for literature exploration and selection were adopted: **(i)** publication should be less than ten years old, **(ii)** it should report on the applied use of molecular tools to denitrifying microbial communities, **(iii)** the oligonucleotide sets should be directed towards *nosZ* in complex microbial communities, **(iv)** the oligonucleotide sets should be cited at least once as an indication of their impact.

As a result, the following publications were selected: Delorme *et al*. (2003), Henry *et al*. (2006), Horn *et al*. (2006), Nogales *et al*. (2002), Rich *et al*. (2003), Rösch *et al*. (2002); Rösch and Bothe (2005) Scala and Kerkhof (1998); Scala and Kerkhof (1999); Throbäck *et al*., (2004).

Data analysis

The orientation of oligonucleotides was tested using FunGene Repository / Pipeline (http://flyingcloud.cme.msu.edu/fungene/). Oligonucleotide pairs were organized according to their use in literature and their *in-silico* melting temperatures (Tm) were calculated according to SantaLucia (1998) using BioEdit 7.0.1 (Hall, 1999). The Tm module was set to calculate the theoretical melting temperature of each DNA oligonucleotide to its exact target site (exact complement) without any mismatches allowed. The environmental parameters during virtual annealing were 50 mM Na^{+}, 2.5 mM Mg^{++} and the concentration of each oligonucleotide was set to 100 nM. The calculation was done using the nearest neighbor thermodynamic model presented by SantaLucia (1998), which was based on the model by Borer *et al*. (1974):

$$Tm = \Delta H / (\Delta S + R*\ln(C/4)) - 273.15$$

where *R* is the molar gas constant and *C* is the concentration of oligonucleotide. A salt correction for ΔS is applied which is: $0.368 \times \ln([Na^{+}])*(P)$ (SantaLucia, 1998), where *P* is the number of phosphates and is equal to *length-1* for non-5'-phosphorylated oligonucleotides such as PCR oligonucleotides. Mg^{++} is assumed to have an effect roughly to 140X the sodium equivalent, according to Nakano *et al*. (1999) and von Ahsen *et al*. (1999).

The calculated Tm were organized to represent each oligonucleotide from a set and for each variant of degenerated oligonucleotide. Further, average Tm and corresponding standard deviations were calculated from the distribution and the differences in Tm among the oligonucleotides used in each set were determined as well. In addition, minimum and maximum Tm of each degenerate oligonucleotide were identified and sorted according to average Tm as primary criteria, and later according to minimum Tm, maximum Tm, fold degeneracy, Tm difference within oligonucleotide pair.

RESULTS AND DISCUSSION

In the present study elucidation of basic relationships between melting temperature, fold degeneracy, differences in melting temperatures of oligonucleotide pairs was conducted based on published oligonucleotide sets from ten studies published during the last decade. The differences in the respective average *in-silico* melting temperatures of oligonucleotides used in source

studies are depicted in Fig. 1. Large differences, up to 12 °C can be observed between predicted average melting temperatures of paired oligonucleotides. Also, three categories can be observed: **(i)** symmetric pairs of two degenerated oligonucleotides (A, I, K, L, M, N, O, P, R, S; n = 10 pairs), **(ii)** asymmetric pairs of degenerated and single variant oligonucleotide (B, D, F, G, H, J; n = 6 pairs) and **(iii)** two symmetric single variant oligonucleotides that were paired in just two cases (C, E). The more frequent use of two degenerated oligonucleotides in pair also reflects attempts of researchers to obtain as comprehensive collection of amplicons from microbial communities as possible.

However, one of the most common approaches during PCR or hybridization optimization procedures is to use developed oligonucleotides and test them on a set of different but defined DNAs. In order to make the two oligonucleotides that differ in average Tm be successful in detection of target genes, Tm of at least one of the oligonucleotides present in pair needs to be violated, either increased or decreased. In case the oligonucleotides are degenerated, this in turn favours binding of certain variants of oligonucleotides to their targets, while some of them can not or do so at much lower stringency, while other perform at optimal or too harsh conditions. This, however, is known to compromise specificity and efficiency of amplification of such approaches. Interestingly, these limitations are expressed in studies optimizing Real-Time PCR assays only. In addition, as the composition of degenerated target sets present in microbial community is not known a-priori, the variants of oligonucleotides may be differentially used up as a function of community structure or differences in chemistry or due to co-extracted impurities. As a result, concentrations of oligonucleotide variants binding to their targets may fall outside the optimal concentrations not allowing uncompromised reaction continuation but leaving only less competitive binding variants to interact with target DNA. As the concentration of most efficient oligonucleotide variant is decreasing during initial PCR cycles in exponential amplification this could in fact change the chemistry of PCR substantially and have impact on the final molecular results as well (Ausubel *et al*., 1999; Morris *et al*., 2002).

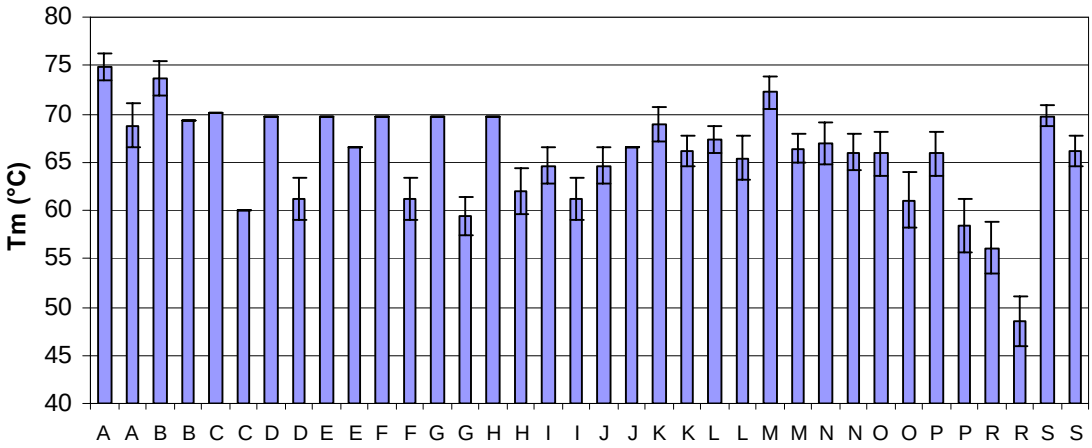

Figure 1. The distribution of average *in-silico* average melting temperatures of oligonucleotides (A-S) adopted from Delorme *et al*. (2003), Henry *et al*. (2006), Horn *et al*. (2006), Nogales *et al*. (2002), Rich *et al*. (2003), Rösch *et al*. (2002); Rösch and Bothe (2005) Scala and Kerkhof (1998); Scala and Kerkhof (1999); Throbäck *et al*. (2004). Tm (°C) – average melting temperature of oligonucleotide.

Slika 1. Porazdelitev povrečnih predvidenih temperatur taljenja začetnih oligonukleotidov (A-S) iz literature: Delorme *et al*. (2003), Henry *et al*. (2006), Horn *et al*. (2006), Nogales *et al*. (2002), Rich *et al*. (2003), Rösch *et al*. (2002); Rösch and Bothe (2005) Scala and Kerkhof (1998); Scala and Kerkhof (1999); Throbäck *et al*., (2004). Tm-povprečna temperatura taljenja začetnega oligonukleotida.

Fig. 2 shows that large differences among Tm are regular practice during oligonucleotide design in microbial ecology as only less than 12.5% of oligonucleotide pairs had predicted difference in average Tm smaller than 2 °C. More than 85% of oligonucleotides had difference in average Tm larger than 2 °C, more than 60% larger than 4 °C and more than 40% larger than 6 °C. Interestingly, 25% of oligonucleotides had Tm difference higher than 8 °C.

It could be argued, that our results are *in-silico* calculations that could hardly hold true if tried in PCR cycler. However, this approach was chosen deliberately in order to control Tm determination and avoid unsystematic technical biases during manipulations such as pipetting errors, unequal mixing, diffusion and chemical decomposition. Therefore, biases in our Tm calculations are systematic for all oligonucleotides. The calculated Tm values thus reflect the differences due to their DNA composition.

Further complicating issue is the generally incompatible chemical composition of PCR or hybridization buffers among different studies, suggesting existence of individual behaviour of oligonucleotide pairs in such mixtures. Surface plasmone resonance could be used to explore this issue (http://www.bf.uni-lj.si/bi/sprcenter/index.html).

Unfortunately, the outcomes of studies deploying different DNA extractions, oligonucleotide sets, PCR reaction conditions, have been subjected to (mis)interpretations many times. In many cases, results from various studies deploying different methodologies have been compared and then some general conclusions were proposed. To avoid comparing apples and oranges, all calculations used in this study applied the same criteria for all tested oligonucleotides.

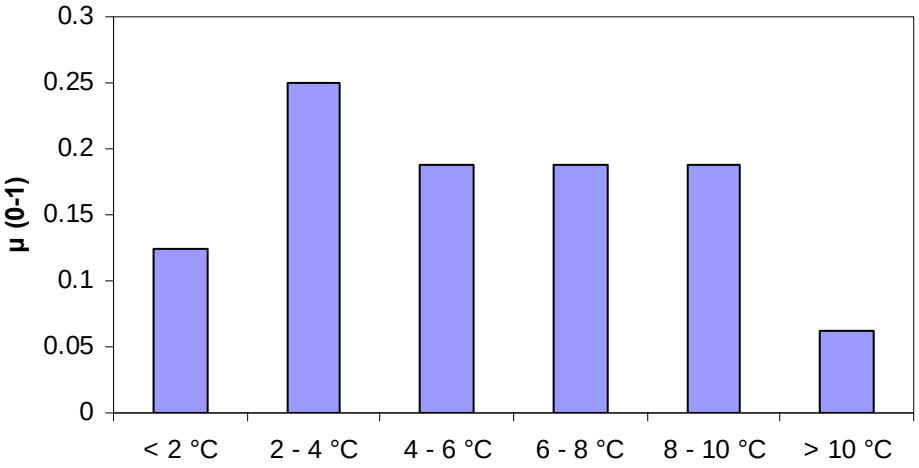

Figure 2. The fraction of oligonucleotide sets (μ) falling into average temperature difference classes: < 2 °C, 2–4 °C, 4–6 °C, 6–8 °C, 8–10 °C, > 10 °C.

Slika 2. Delež oligonukleotidnih setov (μ) porazdeljenih po razlikah v njihovih povprečnih temperaturah taljenja v temperaturne razrede: < 2 °C, 2–4 °C, 4–6 °C, 6–8 °C, 8–10 °C, > 10 °C.

Generally, degenerated oligonucleotide can contain variants with differing melting temperatures for as much as 15 °C. In addition, two degenerated oligonucleotides differing widely (> 8 °C) in their average Tm are routinely being used at one intermediate annealing temperature or using subsequent touch-down protocol (http://www.ncbi.nlm.nih.gov/PubMed). By doing this, the full use of all degenerate variants could never be achieved and consequently brings their specificity, detection limit, the existence and duration of exponential step, efficiency of amplification or hybridization and chemistry mass balances under question.

Of course one can suggest to circumvent PCR step in microbial ecology completely as metagenomic studies and direct reconstructions of genomes from the environment are already on

their course. However, as this is not applicable for majority of research labs in reality, much less drastic approaches can be easily adopted, such as simple adjustment of forward and reverse oligonucleotide melting temperatures or higher concentrations of degenerated oligonucleotides in PCR reactions. The actual sampling capability of each oligonucleotide for target sequences and their specificity were out of scope of our paper.

When the difference between average Tm of paired oligonucleotides (Δ Tm (A-B)) was plotted as a function of difference between minimum and maximum Tm of each oligonucleotide degenerated variants (Δ Tm (min-max)) no observable relationship was observed (Fig. 3). The shaded area designates the Δ Tm (min-max) space occupied by Tm calculated from degenerated variants of single oligonucleotides used in this study (Δ Tm (min-max) = 0 – 13 °C) (Fig. 4). In case all oligonucleotides used in this study were properly paired, their Tm difference between average Tm of each of them would be within 2 °C class and therefore below the horizontal line (Δ Tm (A-B) ≤ 2 °C).

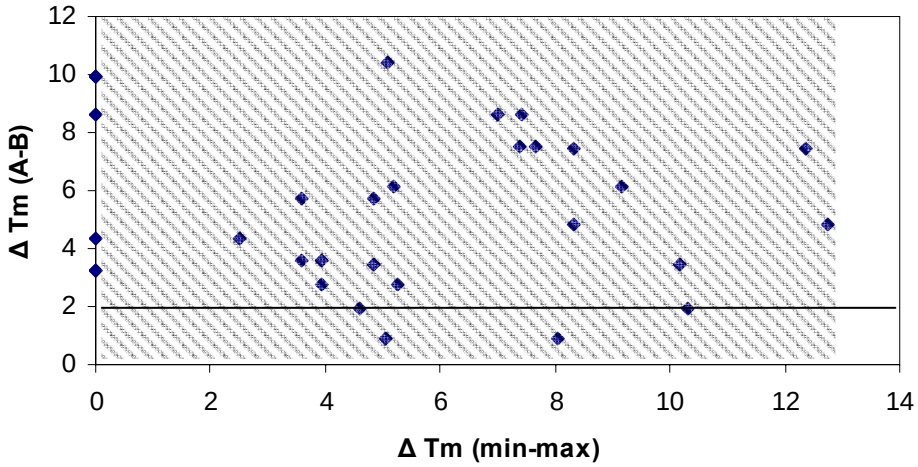

Figure 3. The relationship beween Δ Tm (A-B) (difference between *in-silico* average Tm of each oligonucleotides used in a pair) as a function of difference between maximum and minimum *in-silico* melting temperature of its degenerated variants (Δ Tm (min-max)) of single oligonucleotide.

Slika 3. Odnos med Δ Tm (pair) (razlika med *in-silico* Tm oligonukleotidov v paru) kot funkcija razlike med maksimalno in minimalno *in-silico* temperaturo posameznega od oligonukleotidov (Δ Tm (min-max)).

As this is not the case, this indicates that oligonucleotides are not paired accordingly and therefore violate the basic molecular prerequisites, and yet are in full scientific use. Further, we do not argue that such oligonucleotides would not produce detectable signal when applied to research. However, as shown in Fig. 4, Tm overlap is rarely encompassing hybridization Tm of all forward and reverse degenerate primer variants. Such oligonucleotides and their combinations have been and continue to being used to detect target sequences in complex mixtures of DNA. Therefore, the validity of the subsequent interpretation of molecular signal that is obtained in the form of the comparative diversity of clones sequenced from various environments, or T-RFLP patterns is questioned.

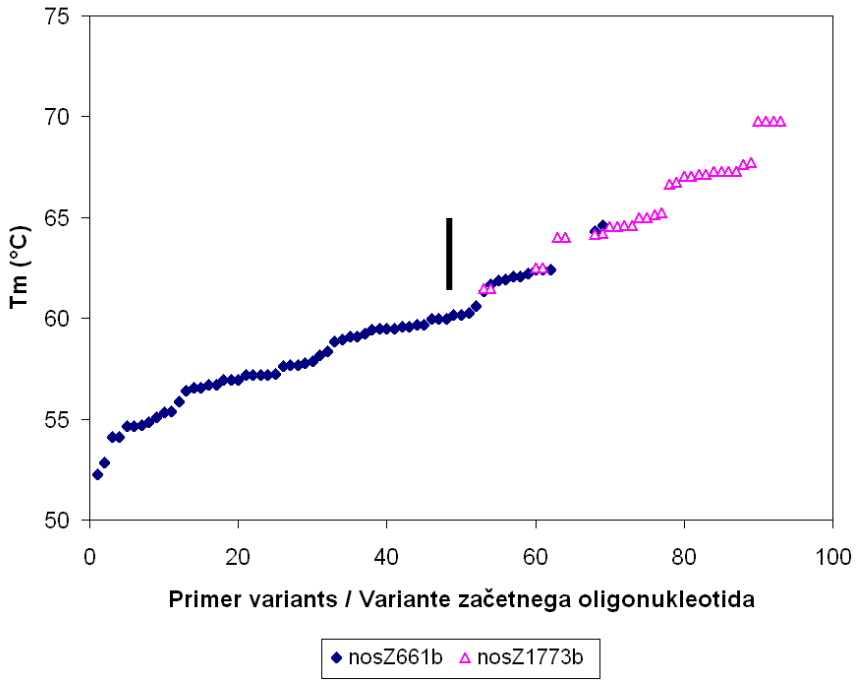

Figure 4. An example of narrow in-silico melting temperature overlap showing nosZ661b (`5′-cggytggggsmwkaccaa-3′`) and nosZ1773b (`5-atrtcgatcarytgntcrtt-3′`) degenerate primer variants. The vertical bar indicates melting temperature overlap of certain nosZ661b and nosZ1773b variants.

Slika 4. Primer ozkega ujemanje izračunanih temperature taljenja dveh degeneriranih začetnih oligonukleotidov nosZ661b (`5′-cggytggggsmwkaccaa-3′`) in nosZ1773b (`5′-atrtcgatcarytgntcrtt-3′`). Pokončna črta označuje ujemanje temperatur taljenja nekaterih variant začetnih degeneriranih oligonukleotidov nosZ661b and nosZ1773b.

CONCLUSIONS

Our analysis of *nosZ* targeting oligonucleotides and the oligonucleotide pairing schemes that are being used in modern microbial ecology all indicated that these approaches underestimate the complexity and vulnerability of research to incomplete, uncontrolled and unaccountable (false) conclusions. Our *in-silico* analysis showed that more than 85% of oligonucleotides had difference in average Tm larger than 2 °C, more than 60% larger than 4 °C and more than 40% larger than 6 °C. Interestingly, 25% of oligonucleotides had Tm difference higher than 8 °C, all indicating thermodynamically unfeasible annealing of a substantial portion of each degenerate primer variants to their respective targets when two highly degenerate primers are paired in single PCR. This issue is of central importance because the results of such studies are projected to be included into important models as novel parameters predicting greenhouse gas emissions that are going to be further delivered to public use for predictive purpose and directing future environmental and economic policies. Looking from that perspective, the issue of compatible melting temperatures should not be taken light-heartedly as rabbit's tail in our hands could hardly be confused for the dragon itself.

REFERENCES

Ausubel, F.M./ Brent, R./ Kingston, R.E./ Moore, D.D. Seidman, J.G./ Smith, J.A./ Struhl, K. Current protocols in molecular biology. New York, John Wiley and Sons, N. Y., 1999.

Borer, P.N./ Dengler, B./ Uhlenbeck, O.C. Stability of ribonucleic acid double-stranded helices. J Mol Biol, 86(1974), 843–853.

Delorme, S./ Philippot, L./ Edel-Hermann, V./ Deulvot, C./ Mougel, C./ Lemanceu, P. Comparative genetic diversity of the *narG, nosZ* and 16S rRNA genes in fluorescent pseudomonads. Appl. Environ. Microbiol., 69(2003), 1004–1012.

Hall, T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser., 41(1999), 95–98.

Henry, S./ Bru, D./ Stres, B./ Hallet, S./Philippot, L. Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG, nirK,* and *nosZ* genes in soils. Appl. Environ. Microbiol., 72(2006), 5181–5187.

Horn, M.A./ Drake, H.L./ Schramm, A. Nitrous oxide reductase genes (*nosZ*) of denitrifying microbial populations in soil and earthworm gut are phylogenetically similar. Appl. Environ. Microbiol., 72(2006), 1019–1026.

Morris, C.E./ Bardin, M./ Berge, O./ Frey-Klett, P./ Fromin, N./ Girardin, H./ Guinebretière, M.-H./ Lebaron, P./ Thiéry, J.M./ Troussellier, M. Microbial biodiversity: approaches to experimental design and hypothesis testing in primary scientific literature from 1975 to 1999. Microbiol. Mol. Biol. Rev., 66(2002), 592–616.

Nakano, S./ Fujimoto, M./ Sugimoto, N. Nucleic acid duplex stability: influence of base composition on cation effects. Nucleic Acids Res., 27(1999), 2957–2965.

Nogales, B./ Timmis, K.N./ Nedwell, D.B./ Osborn, A.M. Detection and diversity of expressed denitrification genes in estuarine sediments after reverse transcription PCR amplification from mRNA. Appl. Environ. Microbiol., 68(2002), 5017–5025.

Rich, J.J./ Heichen, R.S./Bottomley, P.J./ Cromack, K./ Myrold, D.D. Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. Appl. Environ. Microbiol., 69(2003), 5973–5982.

Rösch, C./ Bothe, H. Improved assessment of denitrifying, nitrogen-fixing, and total-community bacteria by terminal restriction fragment length polymorphism analysis using multiple restriction enzymes. Appl. Environ. Microbiol., 71(2005), 2026–2035.

Rösch, C./ Mergel, A./ Bothe, H. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Appl. Environ. Microbiol., 68(2002), 3818–3829.

SantaLucia J, J.R. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. Proc. Natl. Acad. Sci. USA., 95(1998), 1460–1465.

Scala, D.J. / Kerkhof, L.J. Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. FEMS Microb. Ecol. Lett., 162(1998), 61–68.

Scala, D.J. / Kerkhof, L.J. Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments. Appl. Environ. Microbiol., 65(1999), 1681–1687.

Schurgers, G./ Dörsch, P./ Bakken, L./ Leffelaar, P./ Haugen, L.E. Modelling soil anaerobiosis from water retention characteristics and soil respiration. Soil Biol. Biochem., 38(2006), 2637–2644.

Throbäck, I.N./ Enwall, K./ Jarvis, Å./ Hallin, S. Reassessing PCR primers targeting *nirS, nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microb. Ecol., 49(2004), 401–407.

Tiedje, J.M. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In: Biology of anaerobic microorganisms (Ed.: Zehnder, A.J.B. New York, John Wiley & Sons, N.Y., 1988, 179–244.

von Ahsen, N./ Oellerich, M./ Schutz, E. Application of a thermodynamic nearest-neighbor model to estimate nucleic acid stability and optimize probe design: prediction of melting points of multiple mutations of apolipoprotein B-3500 and factor V with a hybridization probe genotyping assay on the LightCycler. Clin Chem, 45(1999), 2094–2101.