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Risk reduction of potential *Vibrio parahaemolyticus* transfer via blue mussels (*Mytilus edulis* L.) into the consumer in the Baltic Sea Region

Reduzierung des Risikos der potentiellen Übertragung von Vibrio parahaemolyticus durch Miesmuscheln (Mytilus edulis L.) auf den Konsumenten in der Ostseeregion

Harvey Harbach[1,2]), Harry W. Palm[1])

Summary

Blue mussels (Bivalvia) potentially accumulate *Vibrio* spp. and can transfer these pathogens into the consumer. Earlier studies dealt with the existence of *V. parahaemolyticus* in natural environments but no examinations under laboratory conditions exist on the influence of temperature onto accumulation and persistence in blue mussels under low salinity environments. The aim of this study was to design a reliable and practicable methodology to examine the presence of *V. parahaemolyticus* in blue mussels from the Baltic Sea and possibilities for decontamination. A static design was chosen, estimating the influence of temperature onto contamination and clearance kinetics. Blue mussels accumulated a similar amount of *V. parahaemolyticus* during 24 h with no significant difference at the chosen temperatures of 5, 10 and 20 °C. After transfer into clearance tanks, the numbers of bacteria decreased in all mussels for 72 h, but the amounts differed significantly between 5 °C to 10 °C and 10 °C to 20 °C. Highest reduction from initial 4.84×10^7 cfu/mg to 1.16×10^5 cfu/mg (72 h) was observed at 10 °C (about 0.2 % of initial value). The Baltic Sea offers opportunities for blue mussel production, especially in the context of Integrated Multi Trophic Aquaculture to reduce environmental impact of fish aquaculture. In the case of mussel infection, a clearance bath for 72 h under 10 °C can prevent possible transfer of *V. parahaemolyticus* into the consumer.

Keywords: Aquaculture, Baltic Sea Region, *Mytilus edulis*, *Vibrio parahaemolyticus*, Depuration, Temperature

Zusammenfassung

Miesmuscheln (Bivalvia) können potentiell *Vibrio* spp. anreichern und diese Pathogene auf den Konsumenten übertragen. Vorangegangene Studien untersuchten die Existenz von *V. parahaemolyticus* in natürlichen Habitaten aber es existieren keine im Labor durchgeführten Untersuchungen zum Einfluss der Temperatur auf die Akkumulation und Persistenz in Miesmuscheln in Umwelten mit niedrigen Salzgehalten. Das Ziel dieser Studie war es eine verlässliche und praktikable Methode zu entwickeln um das Vorhandensein und Möglichkeiten der Dekontamination von *V. parahaemolyticus* in Miesmuscheln der Ostsee zu untersuchen. Ein statisches System wurde ausgewählt um den Einfluss der Temperatur auf die Kontamination- und Reinigungskinetik zu evaluieren. Miesmuscheln akkumulierten ohne signifikante Unterschiede innerhalb von 24 h ähnliche Mengen von *V. parahaemolyticus* bei ausgewählten Temperaturen von 5, 10 und 20 °C. Nach dem Transfer der Muscheln in Reinigungsbecken reduzierte sich über die folgenden 72 h die Anzahl der Bakterien in allen Muscheln, allerdings unterschieden sich die Mengen hier signifikant zwischen 5 °C zu 10 °C und 10 °C zu 20 °C. Die höchste Reduktion von ursprünglich 4.84×10^7 KBE/mg zu 1.16×10^5 KBE/mg (72 h) wurde bei 10 °C beobachtet (cirka 0,2 % des anfänglichen Gehaltes). Die Ostsee bietet Möglichkeiten der Miesmuschelproduktion, im Besonderen im Kontext der Integrierten Multitrophischen Aquakultur um Umwelteinflüsse der Aquakultur von Fisch zu reduzieren. Im Fall der Infektion von Muscheln kann ein Reinigungsbad für 72 h bei 10 °C eine mögliche Übertragung von *V. parahaemolyticus* auf den Konsumenten verhindern.

Schlüsselwörter: Aquakultur, Ostseeregion, *Mytilus edulis*, *Vibrio parahaemolyticus*, Depuration, Temperatur

Introduction

Blue mussels (*Mytilus edulis* L.) are filter feeders of seawater suspended matter and plankton (Storch & Welsch 2004). Depending on the salinity and temperature, the quantity of filtered seawater fluctuates between 5 and 15 liter per individual and day (Gosling 2003). It is well known that the temperature takes influence on the growth rate and activity of poikilothermic animals (Vernberg & Vernberg 1969). The temperature optimum for blue mussels has been determined between 10–20 °C (Coulthard 1929; Widdows 1973). As common filter feeders, they can also accumulate potential pathogens such as belonging to *Vibrio* spp., viruses and also toxic algae (Baker-Austin et al. 2010; Oberbeckmann et al. 2011) and, according to their filter activity, might be differently affected by different sea water temperatures (Gosling 2003). The potential accumulated pathogens can be transferred into the consumer. In the process of cultivation transfer activities of individuals from site to site in different ways and quantities are conducted. With these transfer toxic algae, bacteria and viruses, disease agents are spread and also a mixture of different genetics with unpredictable results may occur (Muehlbauer et al. 2014).

V. parahaemolyticus is one of the most important *Vibrio* species with zoonotic potential (Butt et al. 2004; Gooch et al. 2002; Lee et al. 2003). Reports of foodborne disease outbreaks attributable to *V. parahaemolyticus* contaminated food exist from many countries, either tropical or temperate climate, like for example from Mexico (Cabanillas-Beltrán et al. 2006), Chile (Cabello et al. 2007), Indonesia (Lesmana et al. 2002) and the United States of America (McLaughlin et al. 2005). A number of further foodborne outbreaks have been attributed to *Vibrio* spp. contaminated foods (Chitov et al. 2009; Nguyen et al. 2009). *Vibrio* spp. are highly present in bivalves in general, especially in blue mussels and oysters as well as in other shellfish, crustaceans and fish (Cook et al. 2002; Croci et al. 2001; Lhafi & Kuhne 2007; Ripabelli et al. 1999). With the presence of pathogenic *Vibrio* spp. in crustaceans and bivalves, consumers might be contaminated under consumption of raw or insufficiently cooked crustaceans or bivalves (Dalsgaard et al. 1995; Gopal et al. 2005).

Blue mussels are regularly consumed as food and are one of the favored bivalve species in Europe. It is exploited from natural environments through fishing and also produced in aquaculture and farmed in the North and Baltic Sea. The presence of *Vibrio* spp. in the Baltic Sea water was determined by Eiler at al. (2006), with about log 5 cfu/l, and increasing with raising water temperatures. *V. parahaemolyticus* preferentially grows in brackish waters, which possess less than 30 gL-1 NaCl (Baker-Austin et al. 2010) and in contrast to the well-known risk of the possible uptake and accumulation of pathogens there is little known about factors influencing the uptake and persistence of *Vibrio* spp. in blue mussels, especially under low-salinity conditions (Baker-Austin et al. 2012). The aim of this study was to apply a practicable methodology to examine the presence of *V. parahaemolyticus* in blue mussels. We investigated the influence of temperature onto the uptake and persistence of *V. parahaemolyticus* under brackish water conditions and discuss the consequences for blue mussel aquaculture in the Baltic Sea.

Methods

Blue mussel

Blue mussels originated from a longline culture of the Kiel Fjord, Baltic Sea. The average length of the mussels was 3.28 cm (± 0.58 cm). Animals were transferred into three artificial recirculation systems and were adapted to the examined temperatures 14 days prior to the start of the study. The animals were temperature adapted according to the formula of Baur & Rapp (2003) (temperature difference (°C) x 3 = adaptation time (days)). Surfaces of the closed blue mussels were cleaned from byssus and other adhered materials right before the start of the experiment. Selection of individuals was executed randomly. Mussels are not affected by the contemporary German Animal Welfare Act but the experiments of the laboratory animals followed the rules of the 3-R-principle. Surplus an animal welfare officer accompanied the setup of the different methods.

Artificial system design

The contamination experiments were set up in three aquaria (50 cm x 30 cm x 30 cm) filled with 17 liter artificial sea water ((ASW) Tropic Marine). The aquaria were passive temperated by placing them in water filled wells, which were connected to air condition. The well water was disinfected continuously by potassium permanganate. The salinity was kept at a constant rate of 15 gL-1 as well as the temperature which was adjusted to 5 °C, 10 °C or 20 °C. The three subsequent experiments with 40 specimens each of blue mussels were carried out, with the infection of the mussels followed by the transfer into the decontamination aquaria. This setup avoiding triplicates was chosen in order to allow comments to be made on the distinctiveness of this simple setup, allowing repetition at any other laboratory and field site, e.g. even in mussel cultivating companies. The whole experimental setups were conducted sterile. The aquaria were covered to avoid contamination and a single rotary pump was installed to guarantee water movement and oxygen supply.

1) Uptake of Vibrio parahaemolyticus

At the start of each experiment 40 individuals were introduced into the experimental aquarium at 5 °C, 10 °C or 20 °C, and the *V. parahaemolyticus* bacterial suspension was added ($2{,}63 \times 10^{10}$ to $3{,}5 \times 10^{10}$), corresponding to the final concentration of $8{,}27 \times 10^{8}$ to $3{,}18 \times 10^{9}$. After the uptake period of 24 h samples of mussels (n=6) and water (n=1) were taken in order to determine the presence and amount of *V. parahaemolyticus.*

2) Persistence of Vibrio parahaemolyticus

After 24 h, 28 mussels from each experiment were transferred manually into sterile conditions into the decontamination aquarium with equivalent parameters and equipment, and the corresponding temperature was set to 5 °C, 10 °C or 20 °C under sterile conditions. The mussel surfaces were neither cleaned nor rinsed with fresh water prior to transfer. To ensure suitable water parameters over the experimental time of 72 h after transfer, NH_4, NO_2 and NO_3 values were determined at the beginning and each 24 hours. Over the period of 72 h, samples of mussels (n= 6) and samples of water (n=1) were taken in 24 h intervals to determine the amount of *V. parahaemolyticus.*

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Bacterial cultivation

The isolates of *V. parahaemolyticus* were first grown on Luria-Bertani (LB) agar and incubated at 37 °C for 24 h. They were transferred onto two 2 liter sterile Erlenmeyer flasks containing 400 ml LB medium, incubated at 20 °C ± 2 °C for 3 days at 40 rpm on a rotating shaker. The enriched cultures were centrifuged for 12 min at 10.000 rpm. Supernatants were decanted and the plaques of cells were resuspended in 100 ml sterile 15 gL-1 ASW and pooled in one 1 liter Erlenmeyer flask. The bacterial culture was used for contamination after pooling within 2 hours. The concentration of the bacterial culture was determined in duplicates per treatment and expressed as means.

Statistical analysis

Statistical analysis were conducted with Kruskall-Wallis-Test and using Mann-Whitney-U-test of R for Windows 3.1.0 (R Core Team).

Sample Preparation

Each sampling time six mussels were removed manually with the help of a sterile scoop from the tank and treated individually with sterile articles. The bacterial content of the mussel samples were determined in duplicates and expressed as means per mussel. Blue mussels were opened by cutting the posterior adductor muscle, intravascular water and preparated mussel was collected together. Each individual was homogenized for 2 min with the help of a stomacher and diluted 1:10 with sterile 15 gL-1 ASW. Water aliquots (5 ml) were tested of each sampling in 24 h intervals. The samples were diluted in decimal steps with sterile 15 gL-1 ASW in duplicates. Every thiosulfate-citrate-bile salts-sucrose (TCBS) agar was given a 100 µl aliquot, spread with a drigalski scoop and incubated at 28 °C for 48 h. Colonies were counted and shown as log-colony-forming unit per gram or milliliter (cfu/g or cfu/ml). From each plate, five colonies were verified by Maldi-TOF-MS.

Results

1) Uptake of *Vibrio parahaemolyticus*

The uptake of *V. parahaemolyticus* was determined after 24 h. In all mussel samples (n=6) at all temperatures the amount of *V. parahaemolyticus* increased after 24 h from zero to $1.78x10^7$ cfu/mg at 5 °C, $4.84x10^7$ at 10 °C and $1.27x10^8$ at 20 °C. Figure 1 shows the accumulation of *V. parahaemolyticus* by blue mussels after contamination of the aquarium water, using this simplified experimental setup. There was no significant difference in the mean bacteria counts at the different temperatures. Tendencies were quite similar.

In contrast, the amount of *V. parahaemolyticus* inside the water samples increased during the 24 h at all temperatures, slightly from the initial starting point (5 min) from $8.27x10^8$ cfu/ml to $8.50x10^8$ cfu/ml (+2.8 %) at 5 °C, from $1,10x10^9$ cfu/ml to $1.25x10^9$ cfu/ml (+13.6 %) at 10 °C and from $2.58x10^9$ cfu/ml to $2.94x10^9$ cfu/ml (+14 %) at 20 °C.

2) Persistence of *Vibrio parahaemolyticus*

The examination concerning the persistence of *V. parahaemolyticus* showed a continuous decrease under the three tested temperatures in the water- and the blue mussel samples over the studied period of 72 h (see Figure 2).

The bacteria counts of *V. parahaemolyticus* inside the blue mussel samples decreased 24 h after transfer and further declined continuously over the whole experimental time (72 h). The intensity of decrease of *V. parahaemolyticus* counts in the blue mussels exposed to temperatures differed significantly between 5 °C and 10 °C and 10 °C and 20 °C. At 5 °C the mean *V. parahaemolyticus* contents reduced from $4.77x10^7$ cfu/mg to $1.86x10^6$ cfu/mg at 72 h sampling (4 % of initial value). At 10 °C the mean *V. parahaemolyticus* contents reduced from $5.96x10^7$ cfu/mg to $1.16x10^5$ cfu/mg at 72 h sampling (0.2 % of initial value). At 20 °C the mean *V. parahaemolyticus* counts were reduced

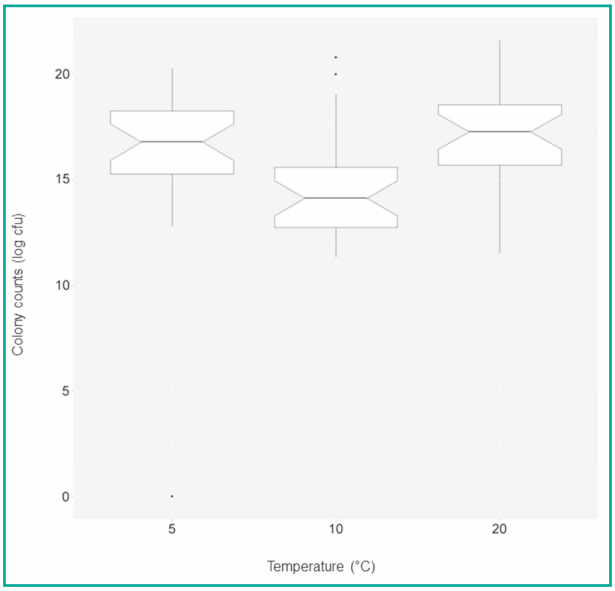

FIGURE 1: *Whisker box plot (medians, quartils, exremes and outliers) of detected V. parahaemolyticus in blue mussel samples (n=6) contaminated for 24 h at temperatures of 5 °C, 10 °C and 20 °C (single examination).*

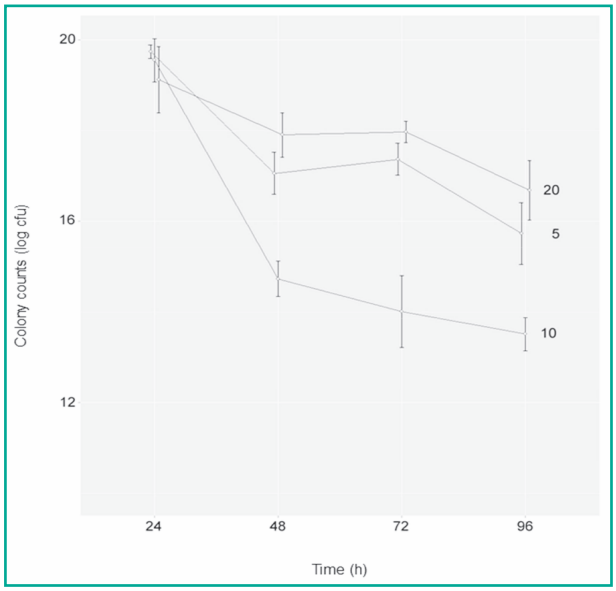

FIGURE 2: *Mean values and corresponding standard deviation of V. parahaemolyticus detected in blue mussel samples (n=6) post contamination at the temperatures 5°Ca, 10°Cb and 20°Ca (a,b: different characters show significant differences between values ($p < 0.05$) single examination).*

from $9.35x10^7$ cfu/mg to $6.19x10^6$ cfu/mg at the 72 h sampling (6.6 % of initial value).

After the transfer of the mussels to the decontamination tank the values of *V. parahaemolyticus* reduced within 24 h inside the water samples to $1.30x10^8$ cfu/ml (15.7 % of the initial value) at 5 °C, to $6.36x10^6$ (0.6 % of the initial value) at 10 °C and to $6.18x10^7$ (2.4 % of the initial value) at 20 °C. In the following sample the values almost remained stable at all tank temperatures, apart from the aquarium with 5 °C temperature. Under this temperature condition the *V. parahaemolyticus* values reduced from the 24 h to 48 h sampling after transfer from $1.30x10^8$ cfu/ml (15.7 % of the initial value) to $1.00x10^7$ cfu/ml (1.2 % of the initial value).

The water parameters inside the decontamination aquarium, namely NO_2, NH_4 and NO_3, were controlled in 24 h intervals and only show minimal increase. After the experimental time of 72 h values of NH_4 was 0.04 mg/l, of NO_2 was 0.07 mg/l and of NO_3 was 1.09 mg/l.

Discussion

This study focused on the uptake and persistence of potentially pathogenic *Vibrio parahaemolyticus* in *Mytilus edulis* under laboratory conditions in brackish water under three different temperatures. This is the first laboratory study focusing on the year cycle temperature range between 5–20 °C at a low salinity of 15 gL-1.

Temperature is one of the most important factors for invertebrate activity in aquatic environments. Vernberg & Vernberg (1969) recorded an influence of the temperature upon growth rate and activity in the crab *Uca* spp. Studies on the influence of temperature upon uptake and effluent of bacteria contents in *M. edulis* and other mussels are scarce. 24 h after contamination of the blue mussels inside the experimental unit *V. parahaemolyticus* enriched inside the tissues (homogenate) under all sampled temperatures (5–20 °C). These findings are in accordance with earlier studies reporting rapid accumulation of *Vibrio* spp. in blue mussels (Herrfurth et al. 2013), clams (Lopez-Joven et al. (2011) and oysters (Murphree & Tamplin 1995). The fast uptake is also in accordance with Gosling (2003), demonstrating the high filtration activity of *M. edulis* between 5–15 liter per day, depending on water temperature and salinity.

Vibrio spp. mostly evades the defending mechanisms of mussels and is able to persist in mussel tissues and hemolymph (Croci et al. 2002; Defer et al. 2009; Hubert et al. 1996; Pruzzo et al. 2005). Accumulation of bacteria in *M. edulis* has been described for the intestine (Töbe et al. 2004; Wang et al. 2010) and the hepatopancreas (Johne et al. 2011). Other studies showed an even distribution of *V. vulnificus* in the gill-tissue, mantle-tissue and hepatopancreas of oysters (Froelich et al. 2010). In several studies on oysters, *Vibrio* spp. remained inside the bivalves up to two weeks (Barile et al. 2009; Ramos et al. 2012; Su et al. 2010; Wang et al. 2010). Studies on *Vibrio* accumulation in mussels mainly dealt with *V. vulnificus* rather than *V. parahaemolyticus.* We have chosen *V. parahaemolyticus* based upon the fact that *Vibrio* spp. can enter a viable but nonculturable (VBNC) state, when they are exposed to unfavorable conditions, e. g. low temperatures. Whereas *V. vulnificus* enters the VBNC state within a period of four days at 5–10 °C (Whitesides & Oliver 1997), *V. parahaemolyticus* is more resilient and enters the VBNC state only within 50–80 days at 3.5 °C (Jiang & Chai 1996). *V. parahaemolyticus* is an extreme tolerant species, occurring at 1–8 gL-1 salinity and reproducing at low temperatures of 8–10 °C (Herrfurth et al. 2013). Consequently, this species was highly suitable for the conducted experiments.

The detected numbers of *Vibrio* spp. in mussels are extremely variable and depend on geographical area, the environmental condition as well as on local parameters. Heidelberg et al. (2002) showed contents of *V. vulnificus* $1.3x10^4$–$1.1x10^7$ liter-1 (largest in summer) without mentioning exact temperatures and salinity values. In invertebrates the concentrations of *V. parahaemolyticus* ranged from <10–12.000 cfu/g in studies from DePaola et al. (2003) with oysters *(Crassostrea virginica),* where the temperature affected the occurrence more than the salinity. Marino et al. (2005) showed for *M. galloprovincialis* no detection of *V. cholera* O1 after 7 days at 14 °C but detection of low numbers at 21 °C in seawater. Unfortunately this study did not include values of *V. cholera* numbers in the surrounding seawater. Tamplin & Capers (1992) showed that *V. vulnificus* reproduced at temperatures above 21 °C in oysters *(C. virginica)* and seawater. Salinity is mentioned as an important parameter to the kinetics of *Vibrio* spp. in the marine system (Hsieh et al. 2008). Several studies indicate that a favored growth and reproduction of *Vibrio* spp. occurs under a decreased salinity (Blackwell & Oliver 2008; Colwell et al. 1977; DePaola et al. 2003; Jiang 2001; Motes et al. 1998; Pfeffer et al. 2003; Randa et al. 2004; Wright et al. 1996).

Eiler et al. (2006) recorded the concentration of *Vibrio* spp. under natural conditions in the Baltic Sea at 10^2–10^5 cfu/ml. This was lower than the tested *V. parahaemolyticus* concentration of $8{,}27x10^8$ to $3{,}18x10^9$. Under these conditions, the uptake rates at the three chosen temperatures differed only marginal, reaching *V. parahaemolyticus* contents inside the mussels from $1.78x10^7$ (5 °C) to $1.27x10^8$ (20 °C) after 24 h. This demonstrates that under availability of high *V. parahaemolyticus* concentrations, these are filtered and directly uptaken. DePaola et al. (2000), Louis et al. (2003) and Venkateswaran et al. (1989) stated the influence of temperature onto existence of microorganisms and clearance rates from mussels. We herewith demonstrate that *V. parahaemolyticus* reduced especially under 10 °C while maintained inside the mussels at 5° and 20 °C in brakkish water. *V. parahaemolyticus* contents in blue mussels reduced after transfer for 24 h into sterile water. This can be explained by a diluting effect, and was demonstrated by the results (2 log stages) of an artificial depuration scenario (15 gL-1 salinity at 15 °C) of blue mussels with *Vibrio* spp. by Herrfurth et al. (2013). In our study the bacteria in the mussels and the surrounding water further reduced without any intervention for 72 h. Another possible reason might be the possible digestion of the bacteria *(V. parahaemolyticus)* by the bivalve (blue mussels) (Arapov et al. 2010).

The developed static experimental design for this study was useful and performed as expected, while being cost efficient and allowing easy maintenance and replicability. Consequently, the chosen experimental setup can be also applied for other potential pathogenic bacteria in future. We avoided triplicates in order to allow determination of differences in the *V. parahaemolyticus* uptake and decontamination in subsequent laboratories with restricted resources and space. This is required in order to apply this methodology even under field conditions. However, all bacterial counts were made in duplicates. According to

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Sutton (2011), total bacterial counts in the water column have a guaranteed preciseness with a mean error of about 0.1–0.2 %, even with non-repetitive but duplicate sampling. The direct-plating method has been reported to enable close agreements between replicate and non-replicate samples in detected *V. parahaemolyticus* levels (DePaola 2003). The TCBS-agar is a selective agar for *Vibrio* spp., guaranteeing *Vibrio* spp. selective growth. Consequently, the chosen methodology of duplicate plating allows the necessary preciseness in terms of bacteria counts within the tested samples while likewise minimizing the required effort in terms of the experimental setup.

We conclude that our results can also be useful for aquaculture producers that can reduce *V. parahaemolyticus* concentrations in *M. edulis* under 10 °C and 15 gL-1 salinity. It would be of highest interest to validate these findings for other food law relevant bacteria species (e. g. *Salmonella* spp., *Escherichia coli*), having consequences for the future use of cultivated blue mussels inside the Baltic Sea and its potential risks for the potential consumers.

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Conflict of interest

The authors herewith declare that they have no conflict of interest.

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