In vivo survey of *Vibrio* spp. in artificially contaminated *Mytilus edulis*

Doreen Herrfurth[1], Keike Schwartz[2], Ralph Pund[2], Eckhard Strauch[2], Thomas Alter[1], Stephan Huehn[1]
1 Institute of Food Hygiene, Free University Berlin, Germany
2 Federal Institute for Risk Assessment, Berlin, Germany

Background

All studies performed on the appearance of *Vibrio* spp. in mussels on pre-harvest and retail level in Germany so far showed high loads with these microorganisms. As far as food safety is concerned *Vibrio* (*V*.) *parahaemolyticus*, *V*. *vulnificus* and *V*. *cholerae* are the most important species within this genus. In a previous study, 106 retail mussel samples of different species and origin were investigated for the presence of *Vibrio*. 54.8 % of the samples were positive for *Vibrio* spp. (approx. 59 % *V*. *alginolyticus*, 12 % *V*. *cholerae* (non-O1, non-O139), 6 % *V*. *parahaemolyticus)*. In addition, single *V*. *metschnikovii*, *Photobacterium damselae* and *Listonella anguillarum* were found.
One of the most frequently consumed mussel species in Germany is the blue mussel, *Mytilus edulis*. This mussel species filtrates approx. 1.5 l of water per hour, thus the surrounding water is clarified effectively (Riisgard, 1988). Along with the water, pathogenic agents, e. g. bacteria (including *Vibrio* spp.), viruses or toxic algae are affiliated. This potential accumulation of *Vibrio* spp. can cause food borne infections if mussels are not properly heated before consumption.
The aim of this study was to determine the clearance kinetics of *Mytilus edulis* after artificial incubation with *V*. *cholerae*, *V*. *parahaemolyticus* or *V*. *vulnificus*. Additionally, the change of *Vibrio* loads in mussels during storage on ice and at 15 ºC was investigated.

Material and Methods

Bacterial strains and mussels
The spiking strains *V*. *cholerae* non-O1/non-O139 (*ctx*$^-$) and *V*. *parahaemolyticus* (*tdh*$^-$/*trh*$^-$) were isolated from wild *Mytilus edulis* while the *V*. *vulnificus* strain was a human isolate, Biotyp 1. For contamination *Vibrio* strains were cultured in alkaline peptone water.
Mytilus edulis mussels used for the experiments originated from the Baltic sea (Kiel Fjord) and were held in artificial seawater at all times.
For all assays the mussels were incubated with *Vibrio* spp. in the contamination unit of the experimental unit (Fig. 1).

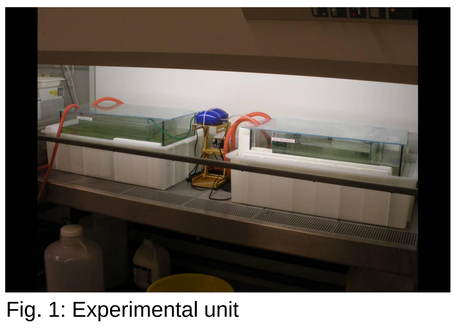

Fig. 1: Experimental unit

Contamination / Bioaccumulation
Overnight cultures of bacterial strains were pooled to 10^{12} CFU for *V*. *parahaemolyticus* and *V*. *cholerae* and 10^{10} CFU for *V*. *vulnificus* which corresponds to a final concentration in the contamination unit of 5 x 10^7 CFU/ml and 5 x 10^5 CFU/ml, respectively. For each experiment approx. 40 mussels were contaminated with *Vibrio* spp. and tested for 24 hours.

Clearance
In clearance assays approx. 100 mussels were transferred after 24 h incubation with approx. 10^{11} CFU (5 x 10^6 CFU/ml) for each *Vibrio* spp. into the husbandry unit to cleanse the mussels from *Vibrio*. The accumulation of *Vibrio* spp. and clearance of mussels was observed over 7 days.

Storage
For the storage test approx. 200 mussels were contaminated with 10^{10} CFU of *Vibrio* spp. which corresponds to 5 x 10^5 CFU/ml in the contamination unit. After 24 hours of incubation mussels were packed in vacuum bags and gunnysacks. A vacuum bag contained five mussels with artificial seawater, whereas each gunnysack contained five mussels only. Samples of both packing strategies were stored for ten days at 3 ºC on ice and at 15 ºC.
Vibrio spp. load was investigated every day for storage at 3 ºC and subsequently every two days for storage at 15 ºC.

Sample preparation
To quantify *Vibrio* spp. the mussels were prepared and subdivided into different sample segments: digestive gland (MDD), lumen water of mussel (IVW), mussel remnants (MR), water of the contamination unit (CV) and husbandry unit (HU) (Fig. 2). Sample processing to quantify *Vibrio* was performed according to ISO/TS 21872.

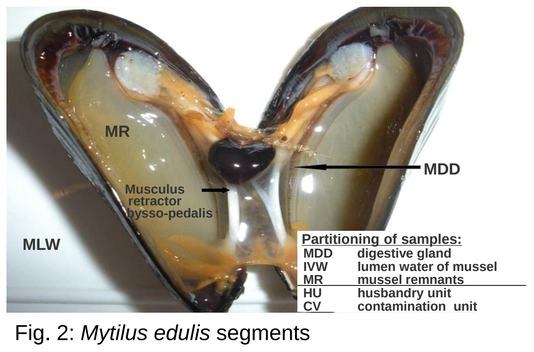

Fig. 2: *Mytilus edulis* segments

fAFLP-Analysis
The fluorescent Amplified Fragment Length Polymorphism (fAFLP) analysis was performed according to Duim *et al.* (1999) except using Taq1 instead of Hha1. This genotyping method was applied to analyse whether the isolated strains at the end of the assay correspond to the spiking strains.

Results

Bioaccumulation assay

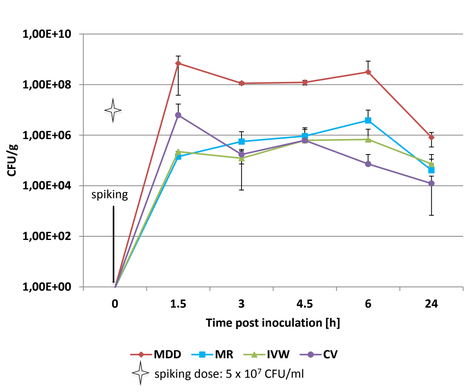

Fig. 3: Cell count of *V. parahaemolyticus* per g *Mytilus edulis* during contamination at 15 ºC for 24 hours.

Vibrio spp. accumulated in *Mytilus edulis* shown by the example of *V. parahaemolyticus* (Fig. 3). Highest enrichment was found after 1.5 h in digestive glands (MDD). Therefore, in the following figures only *Vibrio* levels in digestive glands are shown. Assays with *V*. *cholerae* and *V*. *vulnificus* showed similar results (data not shown).

Clearance assay

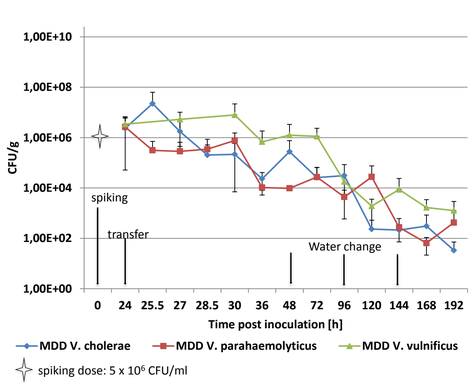

Fig. 4: Cell count of *V. cholerae, V. parahaemolyticus and V. vulnificus* per g *Mytilus edulis* digestive glands (MDD) in husbandry unit at 15 ºC for seven days.

After 24 h of incubation the loads of *V. cholerae and V. vulnificus* were at 10^6 CFU/g, *V*. *parahaemolyticus* loads at 10^7 CFU/g. *Vibrio* cell counts were reduced by 4 - 5 log at 15 ºC to approx. 10^2 – 10^3 CFU/g after 7 days (Fig. 4). To mimic a natural water exchange, 50 % of the water was exchanged with fresh artificial sea water after 48, 96 and 144 h. However, at the end of the clearance period *Vibrio* was still detectable.

Storage assay

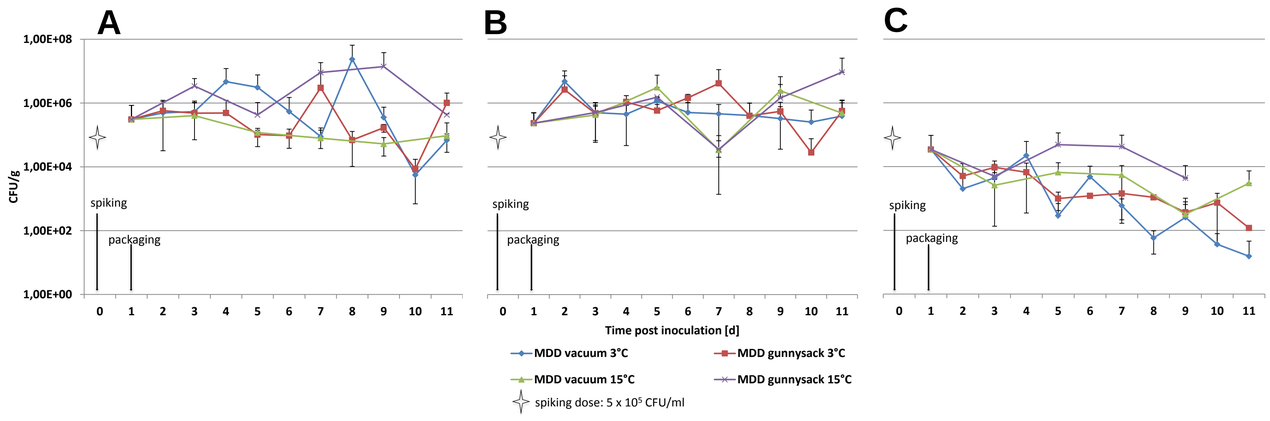

Fig. 5: Cell count of *V. parahaemolyticus (A), V. cholerae (B) and V. vulnificus (C)* per g *Mytilus edulis* digestive glands during storage for ten days.

Starting loads of *V. cholerae* and *V. parahaemolyticus* were approx. 10^5 CFU/g at the beginning of storage (Fig. 5). The concentration was constant at around 10^6 CFU/g with a variance of 2 log during storage for ten days.
The *V*. *vulnificus* load started at 10^4 CFU/g after 24 h of contamination and was reduced gradually from the first day of storage to approx. 10^2 – 10^4 CFU/g .
Among the different storage techniques (vacuum packaging and gunnysacks at 3 ºC or 15 ºC) no differences in the concentrations of *Vibrio* spp. were observed. At the 4th day mussels in gunnysacks at 15 ºC started dying.

Genotyping assay

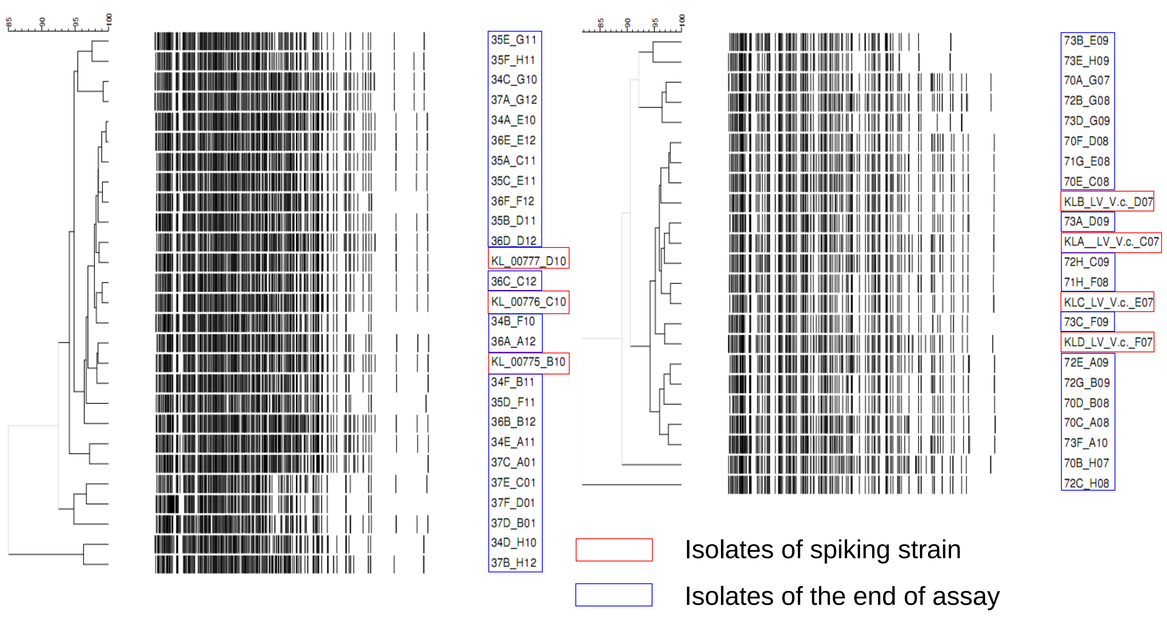

Fig. 6: fAFLP of *V. parahaemolyticus* (A) *and V. cholerae* (B)

To prove that spiking strains and strains isolated from mussels after contamination are identical, fAFLP was performed with selected isolates. fAFLP results before and after storage showed no differences among *V*. *parahaemolyticus* clones (Fig. 6A) as well as among *V*. *cholerae* clones (Fig. 6B).

Discussion

Semi-quantitative methods were used to describe *Vibrio* concentrations within contamination ponds and clearance ponds as well as in mussel lumen water, digestive glands and the remaining mussel meats. The bioaccumulation assays in *Mytilus edulis* showed an enrichment of *Vibrio* especially within digestive glands. After 1.5 h the concentration in the digestive glands was approx. 2 log higher than the corresponding concentration in the tank water. This confirms the 200-fold enrichment described by DePaola *et al*. (2000). At the end of incubation the loads of *Vibrio* spp. were reduced by 1 - 2 log to approx. 10^4 – 10^6 CFU/g in all sample segments. During clearance a slow but continuous reduction of *Vibrio* concentrations was determined within a seven day period in all mussel segments. *Vibrio* loads started in digestive glands at 10^6 CFU/g and were reduced by 4 – 5 log to approx. 10^2 – 10^3 CFU/g in the husbandry unit for seven days. Based on the data, a general clearance rate of 0.023 log-CFU/g per h was calculated (Morisson *et al*. 2011). Once a mussel is contaminated with *Vibrio*, which can occur very likely, even a clearance over a seven day period seems to be insufficient to clear the mussel completely.
Within ten days of storage at 3 ºC on ice or at 15 ºC almost no reduction of the *Vibrio* load occurred in *Mytilus edulis* for *V*. *parahaemolyticus* and *V*. *cholerae*, only variations in a 2 log interval. In contrast, in *V*. *vulnificus* a cell reduction can be seen, probably due to the fact that in the assay a human isolate and no mussel isolate was used. Therefore all *Mytilus edulis* should be thoroughly heated before consumption to avoid potential infection.

References:
Duim *et al.*, 1999, High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with Amplified Fragment Length Polymorphism fingerprinting
DePaola *et al*., 2000, Environmental investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in Washington, Texas, and New York (1997 and 1998)
Riisgard, H. U., 1988, Efficiency of particle retention and filtration-rate in 6 species of northeast american bivalves
Morrison *et al*., 2011, Survival of Salmonella Newport in oysters

Institute of Food Hygiene • FU Berlin • Königsweg 69 • 14163 Berlin • Tel. 0 30 - 838 - 62538 • Fax 0 30 - 838 - 62552 • stephan.huehn@fu-berlin.de • www.vetmed.fu-berlin.de