Norovirus in Pacific Oysters

Insights into Molecular Viability Assays and Mitigation Strategies to Improve Food Safety

Sarah Marie Stoppel

Thesis for the degree of Philosophiae Doctor (PhD)
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SCIENTIFIC ENVIRONMENT

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SAMMENDRAG

Som en av de vanligste årsakene til gastroenteritt, har norovirus (NoV) vært en belastning for pasienter, helsemyndigheter og samfunnet over hele verden. Patogenet overføres gjennom inntak av infektive viruspartikler. Mange matbårne NoV utbrudd har vært knyttet til konsum av rå skalldyr, og østers ser ofte ut til å være en ansvarlig matvare. NoV har vist seg å binde til østersens fordøyelsesvev via histo-blodgruppeantigener, til sialinsyre i gjeller og mantel, og kan til og med finnes i østershemocytter. Derfor akkumulerer østers lett NoV under filtrering og påvirker virusets persistens i perioder med depurering.

For å sikre et trygt produkt er det nødvendig med metoder for å oppdage infeksiøse NoV. NoV er vanskelig å dyrke, så RT-qPCR er den vanligste metoden for NoV-screening, men metoden påviser også RNA fra ikke-intakte virus. Det første målet med dette arbeidet var å finne en effektiv molekylær analysemetode for å påvise intakte og dermed smittsomme virus. For å vurdere kapsidskade ble prøvene behandlet med enzym (RNase) eller interkalerende fargestoff (PMAxx). Når det gjelder genomskade, ble langdistanse PCR-analyse benyttet, siden denne metoden er i stand til å påvise skader i virus RNAet. Ett mål for arbeidet var også å undersøke effekten av ulike virusinaktiveringsmetoder. Det dyrkbare surrogatet Tulane-virus (TuV) ble inkludert i analyser av effekten av varme, UV bestråling og klor. Effekten på dyrkbarhet (infeksjonsevne) ble så sammenlignet med RT-qPCR metoder for å vurdere hvilken molekylær metode som gir det beste målet for virusreduksjon i cellekultur. Hvor godt molekylære metoder gjenspeilte cellekultur var avhengig av inaktiveringsmetode. Reduksjoner på grunn av termisk eksponering og kapsidskade ble best oppdaget hvis PMAxx eller RNase gikk foran RT-qPCR. UV-eksponering, som hovedsakelig fører til genomskader, ble bedre vurdert med en langdistanse-PCR. Åpenbart vil en pålitelig metode som fungerer like godt for alle inaktiveringsmetoder være utfordrende å designe.

Et annet mål med dette prosjektet var å evaluere temperaturutviklingen i østersvev under ulike tilberedningsprosedyrer og å vurdere om disse temperaturene var tilstrekkelige for virusreduksjon. Østers ble tilsatt både NoV og TuV og utsatt for termiske behandlinger. Avhengig av behandling ble smittsom TuV redusert med 1,2 til 3,6 $\log_{10}$. Hvis virusmengden i østers minner om naturlige forurensningsnivåer som beskrevet av *«European Food Safety Authority»* og smittsom NoV reduseres på samme måte som TuV, vil de fleste av de valgte termiske behandlingene resultere i et produkt som er trygt for konsum. Våre RT-qPCR-

resultater indikerer at NoV ble mindre påvirket av de termiske behandlingene enn TuV. Dette illustrerer behovet for en reproduserbar cellekulturanalyse for å korrekt evaluere reduksjoner i smittsom NoV.

Det endelige målet med dette arbeidet fokuserte på modifikasjoner i depureringsprosessen som er en utbredt praksis for å fjerne potensielle patogener fra østers. Det ble undersøkt om klorering av forurensede østers eller en endring i depureringsvanntemperaturen påvirker reduksjonen av smittefarlig virus. For dette formålet ble TuV og/eller NoV bioakkumulert i stillehavsøsters. For å vurdere effekten av klorering ble forurensede østers plassert i sjøvann inneholdende 45 ppm klor i en time. Denne behandlingen hadde imidlertid ingen effekt på infeksjonsevnen til TuV. Flere faktorer kan forklare dette, inkludert tilstedeværelsen av organisk materiale, lokaliseringen av TuV i østersens fordøyelsesvev og den lave temperaturen på sjøvann som ble brukt til klorering (10°C). Å transportere en tilstrekkelig mengde av ethvert antiviralt middel inn i østersens fordøyelseskanal er utfordrende. Som et alternativ til klorering ble effekten av forhøyede vanntemperaturer under depurering evaluert. Kontaminerte østers ble plassert ved 12°C og 17°C og infektivitet og persistens av RNA ble overvåket i en måned. TuV RNA var mer persistent enn NoV RNA. TuV sank med ≤0,7 $\log_{10}$, mens NoV-reduksjoner var ~1,3 $\log_{10}$ ved slutten av depureringsperioden. Muligens reduseres NoV-binding ved samtidig bioakkumulering av begge virus, og sesongmessig ekspresjon av reseptorer i østersen forklare denne forskjellen. Infeksiøs TuV avtok jevnt og det var en signifikant forskjell mellom de to temperaturene. Dette var mest tydelig på dag 14 og 21 da reduksjoner ved 17°C var 1,3-1,7 $\log_{10}$ høyere enn ved 12°C. Etter fire uker ble ikke smittsom TuV påvist ved høyere temperatur, men kunne fortsatt påvises i lave nivåer i 12°C prøver. Lengden på depureringen hadde også innvirkning på reduksjonen i virus. Etter en uke var TuV-reduksjon <1,0 $\log_{10}$, mens reduksjonen ble >4,0 $\log_{10}$ etter fire uker. Dette innebærer at en utvidelse av depureringsperioden til mer enn én uke, muligens i kombinasjon med forhøyede vanntemperaturer, kan være fordelaktig for inaktivering og fjerning av viruspatogener.

Samlet sett illustrerer de presenterte resultatene viktigheten av behandlingsstrategier etter høsting for å redusere risikoen for NoV-infeksjon. Disse strategiene er avgjørende for å motvirke NoV-infeksjoner knyttet til østerskonsum, da naturlig forurensning ikke lett kan unngås i det marine miljøet, spesielt nær strandlinjen. Følgelig er depurering og bruk av antivirale midler fortsatt av relevans.

ABSTRACT

As one of the most common causes of gastroenteritis, norovirus (NoV) has been a burden on patients, health authorities and society worldwide. The pathogen is transferred through ingestion of infectious virus particles. Many food-borne NoV outbreaks have been linked to the consumption of raw shellfish, particularly oysters. NoV has been shown to bind to oyster digestive tissue via histo-blood group antigens, to sialic acid in gills and mantle, and can be found in oyster haemocytes. Thus, oysters readily accumulate NoV during filter feeding, and persistence of the virus during periods of depuration has been demonstrated.

To ensure a safe product, methods to detect infectious NoV are needed. As NoV cannot be easily cultivated, RT-qPCR remains state of the art for NoV screening. Unfortunately, RNA from non-infectious virus is detected as well. The first objective of this work aimed at finding an effective molecular method to primarily detect infectious virus. To assess capsid damage, enzymatic (RNase) and viability dye (PMAxx) pre-treatments were applied. To better assess genome damage, long-range PCR analysis was utilised. The cultivable NoV surrogate Tulane virus (TuV) was exposed to inactivating conditions - heat, UV, and chlorine - to evaluate, which molecular method best approximated virus reductions in cell culture. How well molecular methods compared to cell culture depended on the inactivation mode. Reductions due to thermal exposure and capsid damage were best detected if pre-treatments preceded RT-qPCR. UV exposure, which mainly leads to genome damage, was better assessed with a long-range PCR. A reliable method that performs equally well for all modes of inactivation would be challenging to design.

Another aim of this project was to evaluate temperature development in oyster tissue during various cooking procedures and to assess if these temperatures suffice for virus reduction to safe levels. Oysters were spiked with both NoV and TuV and subjected to thermal treatments. Depending on treatment, infectious TuV was reduced by 1.2 to 3.6 $\log_{10}$. If the virus load in oysters resembles natural contamination levels as described by the European Food Safety Authority and infectious NoV decreases in the same manner as TuV, most selected thermal treatments would result in a product safe for consumption. Our RT-qPCR results indicate that NoV was less affected by the thermal treatments than

TuV. This illustrates the need for a reproducible cell culture assay to correctly evaluate reductions in infectious NoV.

The final objective of this work focused on modifications in the depuration process, which is a widespread practice to remove potential pathogens from oysters. It was evaluated whether chlorination of contaminated oysters or a change in depuration water temperature influences the reduction in infectious virus. For this purpose, TuV and/or NoV were bioaccumulated in Pacific oysters. To assess the effect of chlorination, contaminated oysters were placed in sea water containing 45 ppm chlorine for one hour. However, this treatment did not have any effect on the infectivity of TuV. Several factors may account for this, including the presence of organic matter, the localisation of TuV in oyster digestive tissue and the low temperature of sea water used for chlorination (10°C). Transporting a sufficient amount of any antiviral agent into the oyster digestive tract is challenging. As an alternative to chlorination, the effect of elevated water temperatures during depuration was evaluated. Contaminated oysters were placed at 12°C and 17°C and virus infectivity and persistence of RNA were monitored over the course of one month. TuV RNA was more persistent than NoV. TuV decreased by $\leq$0.7 $\log_{10}$, while NoV reductions were ~1.3 $\log_{10}$ at the end of the depuration period. Possibly, reduced NoV binding during simultaneous bioaccumulation and the seasonal expression of receptors in the oyster may explain this difference. Infectious TuV decreased steadily and there was a significant difference between the two temperatures. This was most evident on days 14 and 21 when reductions at 17°C were 1.3-1.7 $\log_{10}$ higher than at 12°C. After four weeks, infectious TuV was not detected at the higher temperature but was still detectable at low levels in 12°C samples. The length of depuration also had an influence on the decrease in virus. TuV reductions increased from <1.0 $\log_{10}$ after one week to >4.0 $\log_{10}$ after four weeks. This implies an extension of the depuration period, possibly in combination with elevated water temperatures, may be beneficial for the inactivation and removal of viral pathogens.

Overall, the presented results illustrate the importance of post-harvest processing strategies in mitigating the risk of NoV infection. These strategies are crucial in counteracting NoV infections linked to oyster consumption as natural contamination cannot easily be avoided in the marine environment, especially close to the shoreline. Accordingly, depuration and the use of antiviral agents remain of relevance.

LIST OF PUBLICATIONS

PAPER I

Sarah M. Stoppel, Bjørn Tore Lunestad, Mette Myrmel (20XX). The Effect of Enzymatic and Viability Dye Treatment in Combination with Long-Range PCR on Assessing Tulane Virus Infectivity.

Manuscript

PAPER II

Sarah M. Stoppel, Mette Myrmel, Stein Mortensen, Mamata Khatri, Bjørn Tore Lunestad, Arne Duinker (20XX). Temperature Development in Pacific Oysters During Food Preparation Heat Treatments and The Effect on Tulane Virus Infectivity.

Manuscript

PAPER III

Sarah M. Stoppel, Arne Duinker, Mamata Khatri, Bjørn Tore Lunestad, Mette Myrmel (20XX). Temperature Dependent Depuration of Norovirus GII and Tulane Virus from Oysters (*Crassostrea gigas*).

Submitted to *Food and Environmental Virology*.

PAPER IV

Sarah M. Stoppel, Arne Duinker, Mette Myrmel, Bjørn Tore Lunestad (20XX). The Effect of Chlorine on the Infectivity of Tulane Virus as a Surrogate for Norovirus in Pacific Oysters (*Crassostrea gigas*).

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ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CDC	Centers for Disease Control and Prevention
CDF	Chlorine demand-free
cDNA	Complementary DNA
CPE	Cytopathic effect
Ct	Cycle threshold value
dNTP	Deoxyribonucleotide triphosphate
ds	Double-stranded
DT	Digestive tissue
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
FCV	Feline calicivirus
FRNAP	F-specific RNA bacteriophage
FUT2	α-1,2-fucosyltransferase
GC	Genome copies
HAV	Hepatitis A virus
HBGA	Histo-blood group antigen
HBSS	Hanks' Balanced Salt Solution
HIE	Human intestinal enteroids
HPP	High pressure processing
ISO	International Organization for Standardization
kb	Kilobases
Le	Lewis
Lo PCR	Nested one-step long-range RT-PCR and qPCR
Lo RT	Two-step long-range RT-qPCR
LOD	Limit of detection
LOQ	Limit of quantification
MNV	Murine norovirus

MSIS	Norwegian Surveillance System for Communicable Diseases (*"Meldingssystem for smittsomme sykdommer"*)
NA	Non-detects in qPCR
NIPH	Norwegian Institute of Public Health
NoV	Norovirus
ORF	Open reading frame
P1/2	Protruding domain 1/2
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PGM	Porcine gastric mucin
PMA/PMAxx	Propidium monoazide
ppm	Part per million = mg/L
RT-ddPCR	Droplet digital RT-PCR
RTE	Ready-to-eat
RTq	One-step RT-qPCR
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
spp	Species
ss	Single-stranded
$TCID_{50}$	50% tissue culture infective dose assay
TuV	Tulane virus
UV	Ultraviolet
VLP	Virus-like particle
VP1/2	Major capsid protein 1/2
WHO	World Health Organization

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1 INTRODUCTION

1.1 NOROVIRUS

1.1.1 Structure & Classification

The genus *Norovirus* (NoV) is placed in the *Caliciviridae* family. The virus is non-enveloped and possesses a positive-sense, single-stranded (ss) RNA genome. The linear genome is ~7.5 kb in length and contains three open reading frames (ORFs). ORF1 codes for a non-structural polyprotein that is cleaved into several smaller ones required for viral RNA replication (Figure 1). The remaining ORFs 2 and 3 encode the major and minor capsid protein VP1 and VP2, respectively (Jiang et al., 1990; Jiang et al., 1993; Robilotti et al., 2015; Thorne and Goodfellow, 2014). The genomic material is protected by a proteinaceous capsid that provides the virion's structure. The capsid is of icosahedral shape, ~30 nm in size and is formed by 90 VP1 dimers. VP1 carries the shell domain, involved in icosahedron formation, and the protruding (P) domains, P1 and P2. The latter is highly variable among NoV strains, contains putative host receptor binding sites and plays a role in antigenicity (Kniel, 2014; Smith and Smith, 2019).

Based on the amino acid sequence of VP1, NoV is divided into ten genogroups, GI-GX (Chhabra et al., 2019). Each genogroup is further subdivided into genotypes labelled with Arabic numerals. Next to human NoV, viruses infecting other mammalian hosts are included in this classification. Non-human NoV include porcine, feline, murine, bovine, and canine NoV. Traditionally, GI, GII and GIV are associated with human infection, but GVIII and GIX have been established as novel genogroups comprising human NoV (Chhabra et al., 2019; Vinje, 2015). Most abundant, and most relevant in terms of human infection, is GII, especially GII.4, but GI-types are also commonly involved in infections

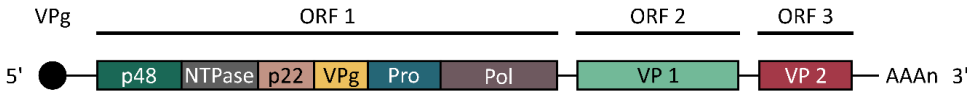

Figure 1: Genome organisation of NoV. The 5' end is covalently bound to the genome-linked viral protein VPg, the 3' end carries a poly(A) tail. The genome comprises three ORFs. ORF2 and 3 encode structural proteins VP1 and VP2, respectively, ORF1 encodes non-structural proteins expressed as a polypeptide. p48/p22 = amino-terminal/3A-like protein, Pro = proteinase, Pol = RNA-dependent RNA polymerase. Figure adapted from Robilotti et al. (2015).

(Kroneman et al., 2008; Verhoef et al., 2010; Verhoef et al., 2009). New variants that first dominate locally and subsequently spread globally emerge regularly. The GII.17 Kawasaki variant is an example for this. It emerged in China in 2014/15, but has since been detected in Japan, the United States and Europe (Chan et al., 2017).

1.1.2 Symptoms & Pathogenesis

Regardless of variant, human NoV may lead to acute gastroenteritis in the infected individual. After a short incubation time of usually <48 h, symptoms may arise. These include diarrhoea and vomiting accompanied by abdominal cramps, nausea, fever, chills, headache, and myalgia. The acute phase of infection lasts for <72 h and symptoms are self-limiting in otherwise healthy adults (Bányai et al., 2018; Devasia et al., 2015; Glass et al., 2009; Robilotti et al., 2015). In the elderly and in children, symptoms can persist for a longer period and the course of infection can be more severe. In these cases, hospitalisation and intravenous fluid replenishment may be required. Fatalities are reported, especially among the elderly. Asymptomatic infections are not uncommon and chronic infections can occur (Lopman et al., 2016; Qi et al., 2018; Trivedi et al., 2013).

The infection process induced by NoV is not completely understood on a cellular level. Ingested NoV particles survive the acidic milieu during gastric passage and reach the small intestine. In the digestive tract NoV binds to histo-blood group antigens (HBGAs) on the surface of epithelial cells (Marionneau et al., 2002). NoV has been detected within epithelial cells morphologically resembling enterocytes, but the role of these cells remains controversial (Green et al., 2020; Karst and Wobus, 2015). In addition, NoV is found in cells of the immune system like macrophages, lymphocytes, and dendritic cells, possibly after transcytosis across enterocytes (Hassan and Baldridge, 2019; Jones et al., 2014; Karandikar et al., 2016; Karst and Wobus, 2015). NoV infection leads to a reduction in villus surface area due to blunting and broadening of the villi. Increased vacuolisation in the mucosal epithelium and apoptosis are observed. Infection also leads to a local inflammatory response with an influx of immune cells and release of cytokines. Transient malabsorption and a reduction in gut motility may contribute to the typical NoV symptomatology (Glass et al., 2009; Hassan and Baldridge, 2019; Karst, 2010; Wobus, 2018).

1.1.3 Susceptibility & Immunity

Binding to HBGAs has received special attention in elucidating the initiation of the infectious process as the receptors seem to play a crucial part in NoV attachment (Graziano et al., 2019; Tenge et al., 2021). HBGAs are terminal structures of oligosaccharides that are attached to the cell membrane via embedded lipids and proteins. The receptors are not only found on mucosal cells and in biological excretions as saliva and milk, but also on red blood cells, determining the ABO blood type (Clausen and Hakomori, 1989; Ravn and Dabelsteen, 2000). It follows that there are specific types of HBGA molecules, type H, A, B and Lewis, as illustrated in Figure 2 (Barbe et al., 2018; Henry et al., 1995; Tan and Jiang, 2005). Depending on NoV genotype, different binding patterns to the various HBGA types are observed (Harrington et al., 2004; Huang et al., 2003; Huang et al., 2005). The increased affinity to specific HBGAs may account for the higher prevalence of some NoV, like GII.4.

To form HBGA molecules on epithelial surfaces, an α-1,2-fucosyltransferase (FUT2) is required. It produces the H type antigen from which other types derive (Figure 2). About 20% of the European population lack this gene and do not express HBGAs in the intestine or in saliva (Wacklin et al., 2011). These individuals are called non-secretors and are often resistant against clinical NoV infections (Currier et al., 2015; Lindesmith et al., 2003; Tan et al., 2008; Teunis et al., 2020). Still, symptomatic NoV infections in secretor-negative individuals and resistance to infection in secretor-positive individuals has been documented (Kambhampati et al., 2016; Lindesmith et al., 2005; Lopman et al., 2015; Rockx et al., 2005). Thus, HBGAs cannot be the only cell receptor of importance in NoV attachment.

As HBGAs are deeply linked to ABO blood type, it has been evaluated whether blood type plays a role in NoV susceptibility. However, a unanimous opinion on this matter cannot be formed. There is no clear evidence that individuals of a specific ABO blood type are more likely to be secretor-positive than others, as the reported frequency of secretors of one blood type differs among publications (Jaff, 2010; Saboor et al., 2014). Some publications suggest that individuals of blood type O are especially prone to GI Norwalk infections, whereas type A and B individuals are less susceptible (Hennessy et al., 2003; Hutson et al., 2002; Lindesmith et al., 2003). NoV GII.4 was also shown to infect more type A individuals compared to type O, but the same was not true for GII.3 (Tan et al.,

2008). Others do not find any connection between symptomatic NoV infection and blood type or secretor status for selected GII strains (Halperin et al., 2008; Lindesmith et al., 2005). This infers the existence of other factors influencing an individual's susceptibility to NoV infections. Overall, there may be a link between NoV infection and ABO blood type, but susceptibility differs greatly and is dependent on NoV variant (Huang et al., 2003; Kambhampati et al., 2016).

Little is known about the immunological response to NoV infections. A human challenge study has shown that NoV infection does not seem to result in long-term immunity. A few weeks after the first encounter with NoV GI, participants were protected from reinfection. However, when challenged with the same strain two years later, symptomatic infections were observed (Parrino et al., 1977). Simmons et al. (2013) estimate the duration of immunity to be much longer, lasting from 4.1 to 8.7 years, based on dynamic data modelling. Serum antibodies against NoV can be detected after an infection but these antibodies may not protect against infection, unless the same or a closely related strain is encountered. Furthermore, human challenge studies suggest no cross-reactive immunity when participants were challenged with a GI and a GII strain, but some cross-reactivity can be observed when challenged with two closely related variants (Lindesmith et al., 2005; Malm et al., 2014; Wyatt et al., 1974).

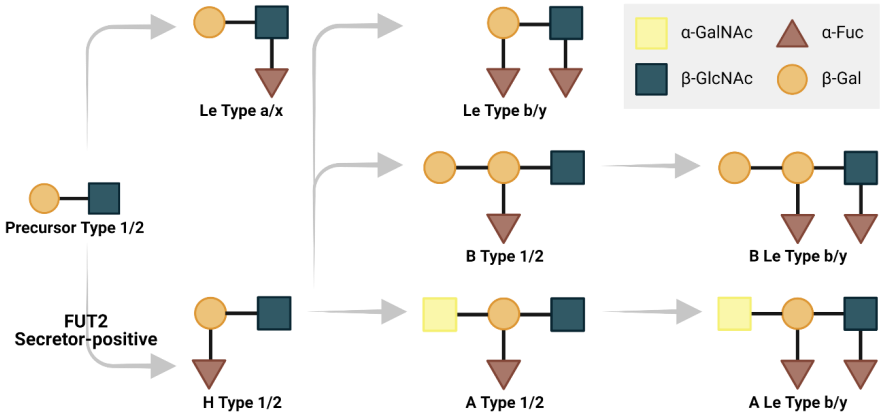

Figure 2: Synthesis pathway of histo-blood group antigens (HBGAs); α-1,2-fucosyltransferase (FUT2) is required to synthesise most of the depicted molecules. Le = Lewis, α-GalNAc = α-N-acetyl galactosamine, β-GlcNAc = β-N-acetyl glucosamine, α-Fuc = α-fucose, β-gal = β-galactose. Figure adapted from Barbe et al. (2018) and created with BioRender (2017).

NoV vaccine development has been challenging. The variability among NoV genogroups combined with the emergence of new variants, the lack of cell culture and animal models, as well as uncertainty about cross-reactivity and long-term immunity contribute to the problem. Vaccine candidates are undergoing preclinical and clinical trials as recently reviewed by Zhang et al. (2021b). Most candidates are based on recombinant virus-like particles (VLPs) of one or two NoV variants. However, none of them will protect against all NoV strains. The majority is supposed to protect against GII.4 or GI.1 (Lucero et al., 2018; Zhang et al., 2021b). Any approved vaccine will likely contain material from the most relevant strains at the time of approval. Vaccine composition will have to be periodically adjusted, depending on the most relevant variant at the time. As NoV in general does not cause severe illness, such a vaccine may only be given to the elderly and immunocompromised who are at risk of severe infections and a fatal course of disease.

1.1.4 Epidemiology & Prevalence

NoV is said to be one of the most common causes of acute gastroenteritis (Bányai et al., 2018; Pires et al., 2015). It may be accountable for 95% of non-bacterial and 50% of all gastroenteritis outbreaks worldwide (Karst, 2010). On a global basis, the annual number of NoV cases is estimated to be 677 million. Deaths due to diarrheal diseases were also most frequently linked to NoV, with >210,000 fatalities annually (Pires et al., 2015). An estimate of 23 million NoV infections may occur yearly in the United States alone (Karst, 2010; Mead et al., 1999). There, it ranks as the most frequent food-borne pathogen, with ~5.5 million confirmed cases annually (Batz et al., 2011). With such a high prevalence, the global economic burden that stems from NoV infection is significant: $4.2 billion direct health system cost and >$60 billion of societal costs have been estimated (Bartsch et al., 2016).

In Norway, the number of confirmed NoV infections registered by The Norwegian Institute of Public Health (NIPH) has been constant in the period from 2008 to 2018 with a yearly average of ~1,500 cases. Sixty-one outbreaks suspected to be NoV-related were reported in 2017. However, these numbers underestimate the occurrence of infection as NoV cases do not have to be reported to the Norwegian Surveillance System for Communicable Diseases (MSIS), except for suspected food-borne and institutional outbreaks. Typically, only larger outbreaks are registered (NIPH, 2018a; b; 2019).

According to the World Health Organization (WHO), developed regions comprise most European and former Soviet countries, The United States and Canada, Japan as well as Australia and New Zealand. High mortality developing regions include countries *"in sub-Saharan Africa, and […] with high child and adult mortality in Asia, Central and South America and the Eastern Mediterranean",* while remaining developing countries are labelled low mortality developing countries (WHO, 2003). NoV is especially prevalent in developed regions and in low mortality developing regions (Ahmed et al., 2014). Given the low number of studies available for high mortality, low-income countries (Ahmed et al., 2014; Mans et al., 2016; Nguyen et al., 2017), this prevalence may change if more data were collected in these regions. In all regions, NoV gastroenteritis is the most frequent cause of diarrheal disease. In terms of deaths due to diarrheal disease, the same can be observed, except in the European region. There, *Salmonella* ranks as the most frequent cause of death across all age groups (Pires et al., 2015). In general, NoV infections occur across all ages. However, infections are especially common in the elderly and immunocompromised patients. In children <5 years, ~70% of cases occur between six and 23 months with a younger age distribution in developing countries (Shioda et al., 2015). In these populations, symptoms can persist longer than usual, and the course of infection can be more severe (Bányai et al., 2018; Lopman et al., 2016).

In temperate climates, NoV infection occurs mostly during winter months (Ahmed et al., 2013; Rohayem, 2009; Steele et al., 2020). The seasonal spike may be due to a combination of factors, including increased rainfall and runoff from land, lower temperatures and humidity preserving virus infectivity and less sunlight that may inactivate viruses in the environment, (Colas de la Noue et al., 2014; Fujioka and Yoneyama, 2002; Lin and Marr, 2020; Shamkhali Chenar and Deng, 2017). A weakened immune system during winter months and an increase in indoor activities may be contributing factors. Seasonality is not necessarily observed in other climates (Mans et al., 2016).

1.1.5 Transmission

As a virus, NoV is dependent on living host cells for replication and is therefore not able to multiply on surfaces or in food and water. The only known reservoir of human NoV is the human intestine. It follows that NoV is spread via the faecal-oral and oral-oral route either directly from person to person via faeces and vomit, or indirectly via contaminated surfaces, food and water (Figure 3). From infected food handlers with poor personal hygiene practice NoV can be transferred onto food products. In case of sewage overflow into a reservoir or if water disinfection systems fail, water can also be contaminated. Ingestion of these contaminated products may lead to infection. This is especially problematic for drinking water and for food that is consumed without additional heating, such as ready-to-eat (RTE) food like sandwiches, fruit, or vegetables. Produce can also be contaminated through irrigation water or fertiliser that harbour NoV. Sewage effluent or runoff from land can transport NoV into the aquatic environment and lead to NoV accumulation in marine organisms like bivalve molluscs (Figure 3). Accordingly, consumption of contaminated shellfish is another route of transmission (De Graaf et al., 2016; Mathijs et al., 2012; Ushijima et al., 2014). Currently, NoV is not recognised as a zoonotic pathogen and the transmission from animals to humans has not been reported. However, zoonotic transmission cannot be fully excluded and should be further investigated, as antibodies against animal NoV can be detected in humans and human NoV can replicate in other mammals, like pigs, dogs, and monkeys (Bank-Wolf et al., 2010; Farkas and Wong Ping Lun, 2014; Sisay et al., 2016; Villabruna et al., 2019; Widdowson et al., 2005).

NoV is known to spread rapidly from one person to the next. In closed environments like retirement homes, kindergartens, cruise ships or military camps NoV is especially prone to spread (De Graaf et al., 2016; Mathijs et al., 2012). This may be due to the shedding of virus particles in faeces, even in asymptomatic cases, and in vomitus of infected individuals (Alsved et al., 2020; Atmar et al., 2008). NoV RNA can still be detected in stool samples for up to eight weeks post infection and may reach a peak of ~10^9 genome copies (GC) per gram faeces, on average four days after being challenged with NoV. Concentrations in vomitus are lower, with a maximum of ~10^6 GC/mL (Atmar et al., 2008; Atmar et al., 2014; Kirby et al., 2016). Even though it should be assumed that not all those virions are

infectious, a low infectious dose makes NoV highly contagious via small droplets formed during an episode of acute gastroenteritis. A 50% infectious dose of ~10^3 GC was observed in human trials, but modelling suggests as few as 18 NoV particles suffice for infection (Atmar et al., 2014; Teunis et al., 2008; Teunis et al., 2020). Virus shedding in faeces also leads to the presence of NoV in sewage, which in turn can lead to the contamination of fertiliser, irrigation water or the marine environment (Bellou et al., 2013; De Graaf et al., 2016; Mathijs et al., 2012). In addition, NoV has been proven to be persistent in the environment and to withstand many common methods applied for antimicrobial reduction like freezing or chemical disinfection. Some resistance to heat treatments and persistence in food has been reported as well (Baert et al., 2009; Cook et al., 2015; Gyawali et al., 2019). Hence, infection via contaminated food is common (Bitler et al., 2013; De Graaf et al., 2016; Mathijs et al., 2012).

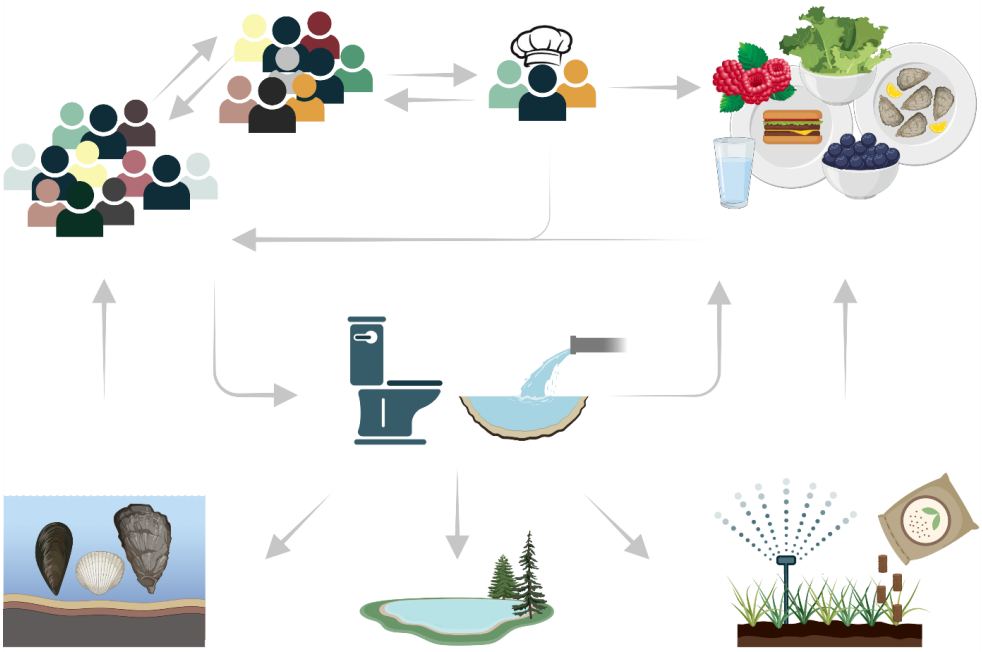

Figure 3: Transmission routes of human NoV. Person-to-person spread is common, and infected food handlers may transfer NoV onto food products. Virus shedding leads to the presence of NoV in sewage, which may lead to the contamination of water bodies and of produce via fertilisers. Faecal contamination of sea water may lead to the contamination of marine organisms. Figure created with BioRender (2017).

1.2 NOROVIRUS & FOOD

1.2.1 Food Safety & Persistence

Food-Processing Strategies

To remove spoilage and pathogenic agents from food products, a variety of strategies are applied in the food industry. Chosen strategies depend on the type of food and microorganism in question. It is common to combine several strategies to achieve the best possible result. Methods of microbial reduction are manifold and the discussion of each one would be beyond the scope of this thesis. Only a few selected approaches are described, but remaining ones are extensively reviewed elsewhere (Baert et al., 2009; Bosch et al., 2018; Cook et al., 2015; Hirneisen et al., 2010; Roos, 2020).

Freezing has a negligible effect on virus infectivity, but elevated temperatures can induce capsid damage in NoV VLPs and lead to the suggested loss of antigenicity and receptor binding capability. The genome is less affected, as genomic material of virus can still be detected even after loss of infectivity due to heat treatment. However, thermal treatment is not a suitable method for all types of food, and the food matrix often has a protective effect, especially fat and protein in shellfish, or milk and sugar (Araud et al., 2016; Croci et al., 2012; Jarke et al., 2013; Park et al., 2015).

Ultraviolet (UV) light may be applied on drinking water, liquid foods, RTE products or food contact surfaces, depending on legislation. In Norway, a minimal dose of 40 mJ/cm^2 should be applied to water to reduce the amount of infectious virus (NIPH, 2012). UV irradiation acts predominantly on the genome, inducing covalent links between adjacent pyrimidines. At high doses, UV can act on the capsid, which has also been observed in RNA viruses (Hirneisen et al., 2010; Smirnov et al., 1983). As with other modes of inactivation, there are factors that influence UV light inactivation. Double stranded genomes are less susceptible to damage and viral aggregation, or a turbid medium may reduce the effect of UV light (Hirneisen et al., 2010).

Chlorine, in the form of sodium and calcium hypochlorite, has traditionally been used in water treatment or on surfaces but has also been applied for washing of produce to eliminate viruses (Casteel et al., 2008; Galal-Gorchev, 1996; Gallandat et al., 2020).

Maximum levels of chlorine differ regionally. For instance, Norwegian drinking water may be treated with ≤5 ppm to reduce the risk of NoV infection (NIPH, 2018c). The mode of action of chlorine on virus is still obscure (Fuzawa et al., 2019; Hirneisen et al., 2010; Wigginton and Kohn, 2012). In poliovirus, RNA cleavage and subsequent RNA release were reported after chlorine treatment, while the capsid could still attach to the host cell and no conformational changes were observed in the capsid (O'Brien and Newman, 1979). After 60 min at 10 ppm chlorine, hepatitis A virus (HAV) maintained antigenicity, even though infectivity has already been lost. At 20 ppm, a loss of antigenicity was observed (Li et al., 2002). The efficacy of chlorine is dependent on pH, temperature, and presence of organic matter. Chlorine acts more efficiently at an acidic pH as the more active dissociation product hypochlorous acid is more abundant. Higher temperatures and reduction in organic load increase the effect of chlorine (Butterfield et al., 1943; Hirneisen et al., 2010; Urakami et al., 2007). As chlorine forms harmful by-products like trihalomethanes when it reacts with organic compounds (Di Cristo et al., 2013; Gil et al., 2019), the use of chlorine in the food industry should be limited.

Persistence of NoV on Food and Food Contact Surfaces

Data on the persistence of NoV on food and on surfaces are readily available. Some years ago, a comprehensive report was published on the matter (Cook et al., 2015). To illustrate NoV persistence, some examples are listed.

NoV was still detected after ten days on refrigerated lettuce and turkey. On berries there was a ≤0.5 $\log_{10}$ decline in NoV at 4-21°C. An exception was a 1.2 $\log_{10}$ reduction on strawberries stored at 21°C for three days. Similarly, a ≤0.5 $\log_{10}$ reduction was observed on various frozen or refrigerated food products, like minced meats and salads. Again, there was one exception when NoV was reduced by 1.7 $\log_{10}$ in potato salad after 24 days of refrigeration (Lamhoujeb et al., 2008; Mormann et al., 2010; Verhaelen et al., 2012). On ceramic, 0.4 to 1.0 $\log_{10}$ reductions were observed after 42 days at room temperature. On formica, often used for kitchen countertops, reductions between 0.6 and 1.5 $\log_{10}$ were recorded after the same amount of time. For stainless steel, $\log_{10}$ reductions recorded after six weeks do not exceed 1.5 $\log_{10}$ in most publications, but a 2.4 $\log_{10}$ reduction after 28 days at 37°C could be noted (Escudero et al., 2012; Liu et al., 2009; Liu et al., 2012).

Even though these findings are based on the persistence of NoV RNA and do not provide information on the infectious state of the virus, food-borne outbreaks after the consumption of contaminated products are common.

1.2.2 Food-Borne Outbreaks

In addition to seafood, fruit, vegetables as well as RTE food products are often implicated in NoV outbreaks. One outbreak affecting at least 74 individuals could be traced back to frozen raspberries that had been used to prepare a dessert dish in Oslo in 2013. Three years prior, the import of contaminated salad led to NoV outbreaks in several regions in Norway (NIPH, 2019). Berries are often associated with NoV outbreaks, for instance in the European Union between 2004 and 2012, or in Germany with >10,000 cases due to contaminated strawberries (Bernard et al., 2014; Callejon et al., 2015). RTE food products like salads, sandwiches, and baked goods can cause NoV outbreaks, and infected food handlers often play a role in transmission. A typical case of poor hygiene involved >300 wedding attendees. Wedding cakes served at the individual receptions were produced at the same bakery at which two employees had previously been infected with NoV (Friedman et al., 2005; Hall et al., 2012; Rönnqvist et al., 2014).

Contaminated water has also been implicated in NoV outbreaks. An outbreak that caused at least 400 cases of NoV infection was recorded in 2001 in Norway. The source of the virus was concluded to be contaminated drinking water (NIPH, 2019). In the time from 2003 to 2012, seven water-borne NoV outbreaks with 277 infected were reported in Norway (Guzman-Herrador et al., 2016). In Nokia, Finland, sewage runoff contaminated a drinking water supply in 2007. Subsequently, >1,000 people sought medical help due to gastroenteritis. Mainly NoV was found in stool samples of infected individuals, but other enteric viruses were present as well (Maunula et al., 2008). Contaminated recreational waters have also been linked to NoV outbreaks, as observed in Finland in 2014 with almost 250 symptomatic cases (Polkowska et al., 2018).

Seafood, in particular bivalve molluscs which are often consumed raw or only lightly heated, is a common cause of NoV outbreaks as elaborated below.

1.3 NOROVIRUS & BIVALVE MOLLUSCAN SHELLFISH

1.3.1 Seafood-Borne Outbreaks

The occurrence of NoV outbreaks after the consumption of seafood dishes is well documented. Including only incidents in Norway, the link between NoV and seafood can be illustrated. After a company Christmas celebration in 2013, 43 of 95 attendees developed symptoms of acute gastroenteritis. Since the only common source of exposure to pathogens was the food consumed during the celebration, a case-control study concluded that infection was due to the consumption of carpet shell clams. The presence of NoV in these clams was confirmed as the causative agent of gastroenteritis (Lunestad et al., 2016). In 2012, at least 41 people fell ill after attending another Christmas celebration at a hotel. Laboratory analysis confirmed that NoV in oysters served during the dinner had been responsible for the outbreak. Additionally, six outbreaks were reported from several restaurants in the Oslo region, all of which were associated with the consumption of imported French oysters (NIPH, 2019). More recently, oysters served at a seafood buffet in northern Norway led to a NoV outbreak (Kristoffersen, 2019).

Shellfish are also listed as the culprit in NoV infections in several publications. A 2017 European Food Safety Authority (EFSA) report focusing on food-borne outbreaks concludes that after *Salmonella* in egg products, NoV in *"crustaceans, shellfish, molluscs and their products"* is the most frequently reported pathogen-food combination. NoV was also responsible for the majority of outbreaks traced back to fish and fishery products (EFSA and ECDC, 2017). In 2013, a review on NoV transmission routes included food-borne NoV outbreaks, of which 65% were linked to shellfish. In comparison, 14% and 22% were associated with produce and RTE food, respectively. In the same year, another review stated that of shellfish-related viral outbreaks, 84% are caused by NoV. Looking at the type of shellfish associated with these outbreaks ~55% were linked to oysters. In 2018, another study concluded that food-related NoV outbreaks are predominantly associated with shellfish (61%). Of these shellfish-associated outbreaks, almost 90% were connected with oysters (Bellou et al., 2013; Bitler et al., 2013; Hardstaff et al., 2018). European Flat oysters have traditionally been important in terms of food safety, but the Pacific oyster is nowadays of special interest, as over the last decades this species has invaded Scandinavian coastlines.

1.3.2 The Pacific Oyster

As other shellfish intended for raw consumption, the Pacific oyster *Crassostrea gigas* (Thunberg, 1793) is frequently linked to NoV infection (Baker et al., 2011; Simmons et al., 2007). The adult bivalve mollusc is a benthic organism living on hard substrates in the littoral zone and feeds on planktonic organisms and detritus suspended in the water by filtration. During summer, gametes are spawned into the water where they develop into pelagic larvae after fertilisation. After several larvae stages, settlement and metamorphosis are induced. Within the first year, the characteristically elongated shells reach a length of ~5 cm and have a riffled and flaky surface (Hovgaard et al., 2001; Troost, 2010).

Invasion

Pacific oysters are natively found in South-East Asia (Troost, 2010) and were introduced to North America already in the 1920s and later in the 1960s and 70s to Europe for the purpose of aquaculture (Grizel and Heral, 1991; Hovgaard et al., 2001; Wrange et al., 2010). In Norway, the oyster was first established in a hatchery in Espevik in 1979 (Strand and Vølstad, 1997). The import of Pacific oysters to Norway for cultivation became restricted in 1986 as the number of cultivated oysters increased to between 2.5 and 3.0 million. From European aquaculture facilities and through passive transport on ships, the Pacific oyster has established self-sustaining colonies (Dolmer et al., 2014; Strand and Vølstad, 1997; Wrange et al., 2010). The first large colony in Southern Norway was found in 2008 on Tjøme, but Pacific oysters may have been present along the Norwegian coast since 2003. The invasive organism is now established as a self-sustaining species in the wild and is found along the Norwegian coast from the Swedish border, with a northbound spread towards Vestland and even further north (Bodvin and Jelmert, 2016; Jelmert et al., 2020) (Figure 4a). The Norwegian Environment Agency (*"Miljødirektoratet"*) also modelled the spread of the Pacific oyster in Norwegian waters and predicts that colonies will permanently establish in these areas (Miljødirektoratet, 2019). This invasion challenges present management models, as the Pacific oyster may be considered both an intruder and a potential new resource, especially for the food industry (Dolmer et al., 2014; Mortensen et al., 2017a; Wrange et al., 2010).

Pacific Oysters as a Safe Food Source

Thus far, the sale of oysters in Norway is negligible compared to other types of seafood like salmon and trout or shrimp and crab. In 2020, the combined sale of European Flat oysters and Pacific oysters (Figure 4b) was worth 930,000 NOK and comprised 20 tons according to the Norwegian Directorate of Fisheries ("*Fiskeridirektoratet*"). In contrast, 1,388,434 tons of Atlantic salmon were sold in the same year. These salmon sales accounted for 64,677,150 NOK (Fiskeridirektoratet, 2021a; b). The Pacific oyster has been established in Norway since at least 2008, and it seems impossible to remove. The most obvious alternative would be to utilize it, for instance as a novel regional food source. If the invasive species could be used as a sustainable food resource, the sale of oysters may contribute more significantly to the Norwegian seafood industry. For this endeavour to be successful, oysters have to be free of pathogens, toxins, and contaminants in harmful concentrations. In terms of algae toxins, levels are mostly below permitted limits and data from the surveillance of blue mussels may be an indicator of toxin contamination. The question of food safety is more difficult to answer with regard to NoV as there is no surveillance program or maximum limit for NoV in oysters (EFSA, 2012; Madejska and Osek, 2021; Mortensen et al., 2019).

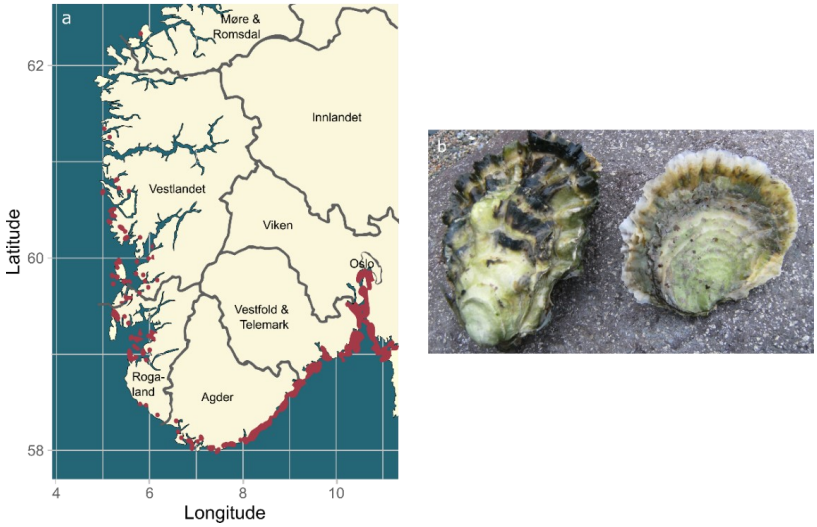

Figure 4: The Pacific oyster: (a) Registered finds of Pacific oysters until 2020 in Norway, map adapted from Jelmert et al. (2020). (b) The Pacific oyster (left) in comparison to the European flat oyster (right); photo by Torjan Bodvin/IMR.

In contrast to bacterial pathogens, like *Vibrio parahaemolyticus*, *V. vulnificus* and *V. cholerae*, NoV is not naturally found in the aquatic environment. It is introduced into the sea by runoff from land, from ships or by sewage and is therefore directly linked to faecal contamination of human origin. Oysters whose habitat is affected by faecal contamination easily accumulate NoV through their filter feeding capability (Bellou et al., 2013; Elbashir et al., 2018; Gyawali et al., 2019; Rippey, 1994). To estimate the level of contamination with enteric pathogens and to help prevent food-borne infections, oyster harvesting areas are categorized according to the level of faecal contamination. To assess the presence of faecal contamination, *Escherichia coli* is used as indicator organism. However, *E. coli* levels do not necessarily resemble the load of enteric viruses (Love et al., 2010; Moqri et al., 2020; Oh et al., 2015a; Sharp et al., 2021). Nevertheless, production areas in Europe are categorised from A to C depending on the number of *E. coli* detected in harvested molluscs. Oysters harvested in a class A area can be directly put on the market, whereas those stemming from B and C areas need to be placed in clean waters so that the number of pathogens in the oyster decreases during relaying or depuration. Alternatively, oysters from B and C areas need to be subjected to a heat treatment (EFSA, 2019). For relaying, oysters are transferred to cleaner areas in the sea, but these areas are often difficult to find within a reasonable distance. Depuration is conducted in land-based facilities in tanks with disinfected sea water (Gyawali et al., 2019; Lees et al., 2010). Due to incidents with infections from oysters from A areas, the industry is occasionally applying these post-harvest treatments to oysters from pristine areas (Rupnik et al., 2018).

1.3.3 Seafood Safety

Shellfish Depuration & NoV Persistence During Depuration

A widespread practice to remove potential pathogens from oysters is depuration. Oysters are placed in tanks with disinfected sea water for typically 48 hours to get rid of contaminants (Lee et al., 2008; McLeod et al., 2017b). This process has been shown to work well for most bacterial pathogens. However, depuration is less effective for viral contaminants, and NoV outbreaks have repeatedly been linked to depurated oysters (McLeod et al., 2017a; Neish, 2013; Sharp et al., 2021; Shen et al., 2019). Allegedly, this is due to the binding of NoV particles to carbohydrate molecules found mainly in oyster digestive tissue (DT). These molecules closely resemble HBGAs to which NoV has been

shown to bind in humans as illustrated in Figure 5 (Huang et al., 2003; Le Guyader et al., 2006; Marionneau et al., 2002; Tian et al., 2006). Additionally, NoV can be detected in oyster connective tissue and in haemocytes. Binding to sialic acids in gills and mantle was also reported for GII strains, but this binding may be linked to NoV elimination from the bivalve (Le Guyader et al., 2012; Lowmoung et al., 2017; Maalouf et al., 2011; Maalouf et al., 2010; McLeod et al., 2017a; McLeod et al., 2017b; Meghnath et al., 2019; Provost et al., 2011; Wang et al., 2008). Hence, the risk for NoV infection even after depuration may be high, especially after consumption of raw oysters (Baert et al., 2009; Cook et al., 2015; Guix et al., 2019; McLeod et al., 2017b). To correctly assess the hazard of NoV in oysters, it is crucial to determine whether the accumulated virus particles remain infectious.

The persistence of NoV within oysters during depuration is well documented. Contrary to *E. coli* that can already be removed from oyster tissue within 24-48 h, such short depuration times are not efficiently removing NoV (Battistini et al., 2021b; McLeod et al., 2009; Neish, 2013). Depuration times of up to ten days lead to the removal of some NoV, but the decrease in virus is often negligible. NoV was reduced by 0.5 $\log_{10}$ after four days and did not decline further in a nine-day period (Battistini et al., 2021b). After a three- and seven-day depuration, GI and GII were reduced by 0.4 and 0.8 $\log_{10}$ on average (Rupnik et al., 2021). A GI strain was also only reduced by 0.5 and 0.9 $\log_{10}$ after eight days of depuration (Drouaz et al., 2015). It may be that even a ten-day depuration does not lead to any decrease in GII (Ueki et al., 2007). It has been observed that NoV persists for several weeks and can often be detected after more than one month. NoV was relatively stable over a two-week period and was also detected after up to four weeks, depending on oyster species

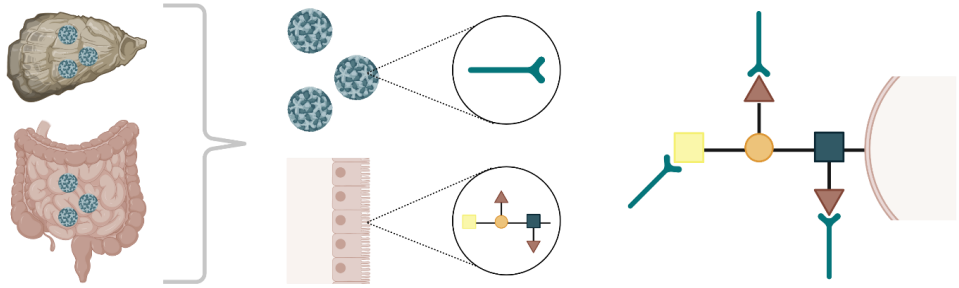

Figure 5: Structures in the P domain (—<) on the NoV capsid bind to HBGA-like receptors on the surface of oyster digestive tissue and the human intestine. Figure created with BioRender (2017).

(Nappier et al., 2008; Nappier et al., 2010; Neish, 2013). An extended period of six weeks was also not able to eliminate NoV (Choi and Kingsley, 2016). Another study showed the reduction in NoV below the limit of quantification after eight weeks (Drouaz et al., 2015).

As commercial depuration is often unable to prevent NoV infections associated with oyster consumption, modifications have been applied to the depuration process. These should improve the removal of viral pathogens from oyster tissue. The effect of light, feeding, salinity and water disturbance has been examined. Also, different modes of water disinfection have been applied and depuration times have been extended. Another approach may be elevated water temperatures during depuration (Battistini et al., 2021b; Drouaz et al., 2015; Neish, 2013; Polo et al., 2018; Rupnik et al., 2021; Younger et al., 2020). Temperatures as low as 8°C may be used during oyster depuration in the United Kingdom. Depuration temperatures vary depending on geographic region and reach even 25°C in China and parts of Australia (McLeod et al., 2017b). At an increase in water temperature up to 28°C, oyster filtration rate, oxygen consumption and enzymatic activity are shown to increase (Brock et al., 1986; Hutchinson and Hawkins, 1992; Kim, 1995; Numaguchi, 1994; Sytnik and Zolotnitskiy, 2014). Lower temperatures lead to low metabolic activity in the oyster and seem to decrease virus removal (Lees et al., 2010). Therefore, the elimination of viruses may increase at higher water temperatures. There is evidence that raising water temperature may increase pathogen elimination. For instance, *Vibrio* spp. were more efficiently removed from oysters at 15°C than at 10°C and 5°C (Chae et al., 2009). For virus removal, there may also be a benefit of elevated temperatures (Neish, 2013; Rupnik et al., 2021; Younger et al., 2020).

It has also been suggested to find agents that break the NoV-HBGA linkage to enhance depuration efficacy. In a preliminary study, proteinases, amylases and oxidizing compounds have been investigated and suggestions for further research have been made (McLeod et al., 2017b). The use of essential oils and other plant extracts embedded in alginate microbeads might be another way to target NoV in oysters (Ryu et al., 2015). Alginate beads are taken up by *Ostrea edulis* and are transported into the DT as well as into haemocytes (Darmody et al., 2015). Therefore, antiviral agents or enzymes released by filtrated bacteria might inactivate NoV during depuration and make its presence in oysters intended for consumption harmless (Fajardo et al., 2014; McLeod et al., 2017a).

Shellfish Processing Strategies

As the removal of NoV from shellfish tissue can be challenging, processing strategies of contaminated oysters that may facilitate the reduction in virus infectivity have been investigated. Refrigeration or freezing would be the easiest approach, but neither seems to be effective. NoV RNA was only reduced by 1.0 $\log_{10}$ during a 120-day period at -80°C and 14 freeze-thaw cycles. Refrigeration at 4°C reduced infectious HAV by 1.7 $\log_{10}$ over a course of 28 days (Hewitt and Greening, 2004; Richards et al., 2012). The product would have long expired before enteric viruses would be completely inactivated at refrigeration temperatures.

Instead of lowering the temperature, oysters could be subjected to heat treatments prior to consumption. Unfortunately, viruses have a reputation of being resilient towards thermal inactivation in food matrices (Araud et al., 2016; Baert et al., 2009; Croci et al., 2012; Jarke et al., 2013; Park et al., 2015). Moreover, the shellfish itself has a protective effect on viruses. Viruses in bivalves maintain infectivity at conditions that inactivate those diluted in PBS. NoV decreased by ~3.0 $\log_{10}$ after 6 min at 80°C, but no such reduction is detected in spiked mussels (Croci et al., 2012). The same study shows that feline calicivirus (FCV) dilutions are no longer infectious after 3 min at 80°C, whereas in mussels FCV is still infectious in cell culture. Abalone adductor muscle and viscera also have a protective effect on infectious murine norovirus (MNV) (Park et al., 2015). Similarly, combinations of 70°C/20 min, 80°C/10 min and 90°C/5 min are suggested to achieve a >1.0 $\log_{10}$ reduction in NoV RNA in homogenized mussels (Jeon et al., 2020). Flannery et al. (2014) conclude that an F-specific RNA bacteriophage (FRNAP) in mussels is no longer infectious after >3 min at a water temperature of >90°C, but remains infectious at 70°C. To inactivate Tulane virus (TuV) and MNV in oyster tissue, 3 min at 80°C were necessary and MNV needed ≥3 min at 90°C for inactivation in clams (Araud et al., 2016; Sow et al., 2011). In contrast, Shao et al. (2018) reported that 63-67°C for 1 min inactivate TuV and MNV in oyster homogenate. Overall, most studies indicate that temperatures >85°C should be applied for 3-5 min to make virus-positive bivalves safe to eat. However, this might lead to a less acceptable product with a decline in organoleptic properties due to prolonged heating.

An alternative to thermal treatment is high pressure processing (HPP). Applied pressure leads to an influx of water from shellfish into the virus which results in capsid distortion (Gyawali et al., 2019). An advantage is the low temperature and the homogenous application of the HPP treatment. Just as with thermal treatments, virus in shellfish is more difficult to inactivate than virus in buffer (Takahashi et al., 2019). The process is also dependent on pH, salinity and temperature and has shown varying success in virus inactivation. NoV RNA was reduced by 3.0-4.0 $\log_{10}$ after HPP at 400-500 MPa for 2 min, but a human challenge study indicates that 600 MPa is necessary to prevent NoV infection (Leon et al., 2011; Li et al., 2009; Li et al., 2013). Furthermore, HPP is not cost-efficient and has a negative impact on oyster tissue, leading to softening and drying. Reduced shelf-life and unpleasant odours have also been reported (Gyawali et al., 2019), whereas another study found no negative impact of HPP on sensory characteristics (Ye et al., 2015).

Limitations of NoV Data Interpretation

Data on NoV persistence has been generated by reverse transcription quantitative PCR (RT-qPCR). This method does not give any information on virus infectivity, so it is possible that although NoV genomic material persists, the virus is no longer infectious. To evaluate the efficacy of the described depuration and shellfish processing strategies in reducing the load of infectious NoV, it is crucial to determine the number of infectious viruses in oyster tissue. Usually, cell culture-based methods are applied to determine infectious virus numbers, but this has been challenging for NoV. Successful *in vitro* cultivation attempts have been made but RT-qPCR remains the gold standard in detecting NoV in food samples. Advancements in NoV cultivation may help determine the loss of infectivity during depuration and post-harvest processing. Several attempts have been made to cultivate NoV in different cell types. A promising approach in NoV cultivation was the replication of the virus in human intestinal enteroids (HIE) and B cells (Ettayebi et al., 2016; Jones et al., 2014). Despite these advancements, none of those systems has been routinely established as a reproducible assay to determine NoV infectivity (DiCaprio, 2017; Manuel et al., 2018).

1.4 VIRUS DETECTION

1.4.1 Cell Culture Methods

To quantify infectious virus, cell culture-based methods are generally utilized. Common are plaque assay and 50% tissue culture infective dose assay ($TCID_{50}$) that both work with serial virus dilutions. In plaque assay, virus dilutions are added to a specific cell line. Infection of those cells leads to cell lysis, resulting in empty plaques in the monolayer stemming from a single virus particle. An overlay medium is added which solidifies on top of the infected cells. Thus, newly released viruses can only infect neighbouring cells and are unable to migrate. For the determination of virus titre, samples are fixed and stained so that plaques can be counted. The infectious virus titre is given as plaque forming units per mL. In $TCID_{50}$, susceptible cell lines are also inoculated with virus dilutions, but no overlay medium is added. Viruses quantified by $TCID_{50}$ often induce changes in cell morphology. This cytopathic effect (CPE) manifests in different ways, depending on virus and cell type, and includes detachment from the monolayer, rounding of cells and the formation of syncytia. After incubation, wells that show CPE are noted and used to calculate $TCID_{50}$ per mL. The results resemble the dilution of virus that infects 50% of the inoculated cells. In addition to those assays, other methods of quantification of infectious virus are for example fluorescence or hemagglutination assays (Hierholzer and Killington, 1996).

1.4.2 Molecular Methods

PCR Analyses

Despite advancements in recent years, no routine cell culture assay for the detection of infectious NoV has been established. Alternatively, NoV is detected by RT-qPCR. Viral RNA is reverse transcribed into cDNA, which in turn is amplified in a qPCR reaction. As in traditional PCR, the target is amplified during cycles of denaturation, annealing and extension (Figure 6). In qPCR, amplification leads to an increasing fluorescent signal that facilitates template quantification. Fluorescence is emitted by unspecific dyes, like SYBR green, or by labelled probes that specifically bind to the target. As dyes like SYBR green bind to all double-stranded (ds) DNA, assay specificity is low, and an additional melting curve analysis is needed. Increasing the temperature denatures the amplicon so that SYBR

green is released. The fluorescence decreases and is plotted over temperature. Since each dsDNA possesses a specific melting temperature, the desired target can be distinguished from unspecific amplicons. The use of short ssDNA probes that carry a fluorochrome and a quencher on opposite ends is the more specific approach. The probe binds to the target sequence during annealing but is displaced and hydrolysed during elongation (Figure 6). The fluorochrome is released and emits a light signal that can be detected.

Results are given in cycle threshold (Ct) values. They resemble the cycle number at which the detected fluorescence exceeds the set threshold (Figure 6). The more genomic material is detected, the lower the Ct value will be and vice versa. If a standard curve is generated with virus reference material of a known concentration, virus in analysed samples can be quantified by extrapolating concentrations from the standard curve (absolute quantification). Alternatively, Ct values can be compared to those of a reference sample to obtain a relative difference in gene expression (relative quantification), omitting the need for standardised reference material (Schmidt and Rothhämel, 2012).

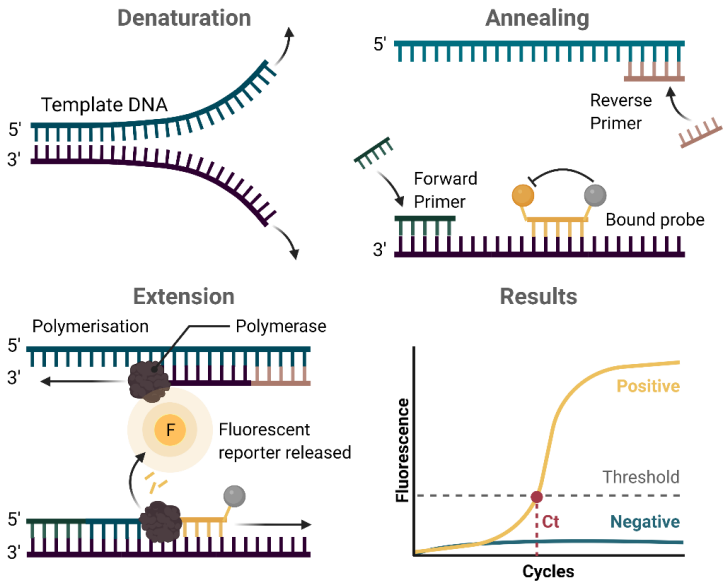

Figure 6: Principle of probe-based qPCR. Double-stranded DNA is denatured into single strands, primers and probe bind to the template (annealing) and complementary strands are formed (extension). Adapted from "Fluorescent Probe-Based Real Time PCR" by BioRender (2022).

For NoV and HAV detection in food products such as fruit, vegetables, bottled water, and bivalve molluscan shellfish, RT-qPCR as described in ISO 15216-1 remains state of the art but does not provide information on virus infectivity (ISO, 2017; Lowther et al., 2019; Richards, 1999). In addition, RT-qPCR has been shown to be negatively affected by inhibitors present in the food matrix. For seafood, algae, glycogen, and other polysaccharides are relevant inhibitors (Schrader et al., 2012; Suther and Moore, 2019). Droplet digital RT-PCR (RT-ddPCR) is an alternative to RT-qPCR, and this method is not as strongly influenced by inhibitors as qPCR. In RT-ddPCR the sample RNA is dispersed into small oil droplets. In every single droplet RNA is reverse transcribed and amplified in RT-PCR. The PCR product in each droplet is detected with help of a fluorescent signal. The number of droplets with a positive fluorescence correlates with the amount of product. Not only is this method less affected by inhibitors in the sample, but ddPCR is independent of PCR efficiency. Moreover, ddPCR is more sensitive than qPCR, and absolute quantification is possible without a standard curve (Hindson et al., 2011).

Approaches to Assess Viral Infectivity

As distinguishing infectious from non-infectious NoV is impossible with qPCR alone, other strategies have been explored, which aim to detect virus capsid or genome integrity. One method is utilizing the NoV-HBGA linkage that is supposedly only formed by infectious NoV. HBGA-like structures are present in porcine gastric mucin (PGM). PGM is bound to magnetic beads and intact NoV will attach to these molecules *in vitro* (Dancho et al., 2012; Tian et al., 2005). Virus binding to cell lines can also be utilized to assess virus infectivity (Li et al., 2012; Rachmadi et al., 2018). The application of aptamers, small ss nucleic acids that bind to NoV due to their secondary structure, are another approach. (Giamberardino et al., 2013; Moore et al., 2016; Weerathunge et al., 2019). Intercalating agents like propidium monoazide (PMA/PMAxx) that penetrate disrupted capsids and bind to the genome are applied as well. In case of intercalation, PCR amplification is inhibited and non-infectious NoV should give a reduced signal in RT-qPCR (Nocker et al., 2006; Parshionikar et al., 2010; Randazzo et al., 2018; Razafimahefa et al., 2021; Sarmento et al., 2020). Moreover, a proteinase K/RNase pre-treatment can be applied prior to qPCR and should degrade both defective capsids and unprotected RNA (Nuanualsuwan and Cliver, 2002).

Attempts have been made to assess virus infectivity by examining genome integrity. For this purpose, the target sequence in PCR analysis can be extended (Ho et al., 2016; Li et al., 2014; McLellan et al., 2016; Rodriguez et al., 2013; Wolf et al., 2009). In RT-qPCR short amplicons, usually ≤100 bp, are obtained, which can occur even if other genomic regions may be damaged. This can lead to false-positive results regarding infectivity. In long-range PCR, the target sequence is extended to several hundred bp to >1 kb. Therefore, genomic damage is more likely to fall within the target region and, ideally, reduced amounts of the amplicon should be generated. False-positive signals should thus be reduced.

1.4.3 Surrogate Viruses

Unfortunately, none of the above-mentioned techniques are completely reliable. For this reason, it is a widespread practice to use related viruses, such as MNV, FCV, or FRNAP, for example MS2, as surrogates (Kniel, 2014). These viruses can be evaluated both in RT-qPCR and in *in vitro* assays, utilizing cell lines or, in case of bacteriophages, bacterial hosts. TuV (Farkas et al., 2008) has been suggested as another alternative to study NoV in oysters. It has been shown that TuV is more similar to NoV than traditional surrogates when it comes to binding to HBGAs (Table 1). Persistence of TuV RNA in oysters during depuration as well as tissue distribution can also be compared to that of human NoV (Drouaz et al., 2015; Farkas et al., 2010; Kniel, 2014; Polo et al., 2018; Tan et al., 2015; Zhang et al., 2015). Like NoV, TuV belongs to the *Caliciviridae*, but represents a new genus (*Recovirus*), a sister groups of the *Norovirus* genus. TuV was first isolated from stool of rhesus macaques (*Macaca mulatta*), and can be propagated in LLC-MK2 cells, a monkey kidney cell line (Farkas et al., 2008). The TuV genome is shorter than that of NoV (Table 1) but is similarly structured. Even though TuV is not recognised as a human pathogen, anti-TuV antibodies have been detected in human serum (Farkas and Wong Ping Lun, 2014). TuV persistence on surfaces as well as in surface and tap water has been demonstrated (Arthur and Gibson, 2016). In these two types of water, TuV was reduced by ≤0.5 $\log_{10}$ after 28 days, whereas in ground and distilled water reductions ranged from 2.2 $\log_{10}$ to 3.5 $\log_{10}$. On surfaces, TuV decreased by <1.0 $\log_{10}$ within 14 days. Overall, TuV may be a good alternative for the study of NoV due to similarities in structure, epidemiology, and additional properties as elaborated by Farkas (2015). It must be acknowledged that it is not necessarily possible to extrapolate from data on surrogate viruses to NoV itself.

1.4.4 Virus Recovery from Seafood Matrices

To recover virus from seafood samples, two methods are commonly used. An extraction method relying on enzymatic treatment of the food sample is described in ISO 15216-1. The method is based on a proteinase K treatment in combination with a 37°C and a 60°C thermal incubation (ISO, 2017). During this process, the capsid is damaged so that virus infectivity is negatively impacted (Langlet et al., 2018; Nuanualsuwan and Cliver, 2002).

The other common extraction is the elution-concentration method. In a first step, virus particles are released from the food matrix with the help of buffers. More alkaline solutions facilitate virus detachment, whereas acidic solutions increase virus attachment. Various components can be added to improve virus recovery and are reviewed in Stals et al. (2012). For instance, beef extract can improve subsequent polyethylene glycol (PEG) flocculation, and glycine hinders non-specific absorption of virus particles to food. After elution, viruses need to be concentrated as they are usually present in small numbers in the food matrix. For this purpose, Cat-Floc and PEG precipitation, ultrafiltration or ultracentrifugation can be applied, among others. After elution and concentration, samples can be further purified. Chloroform:butanol or filtration are among the possibilities (Atmar et al., 1995; Goyal and Aboubakr, 2016; Razafimahefa et al., 2020; Stals et al., 2012).

Table 1: Comparison of NoV with its most common surrogates feline calicivirus (FCV), murine norovirus (MNV), Tulane virus (TuV) and MS2 phage. ⌀ Diameter; (a) Virus shedding in the respiratory tract is reported; (b) as reported by McFadden et al. (2011). Adapted from Kniel (2014).

	NoV	**FCV**	**MNV**	**TuV**	**MS2**
Family	*Caliciviridae*	*Caliciviridae*	*Caliciviridae*	*Caliciviridae*	*Leviviridae*
Genus	*Norovirus*	*Vesivirus*	*Norovirus*	*Recovirus*	*Levivirus*
Replication	Intestinal tract	Respiratory tract	Intestinal tract	Intestinal tract	*E. coli*
Shedding	Yes	No (a)	Yes	Yes	No/Yes
Envelope	No	No	No	No	No
Icosahedral Capsid ⌀	27-38 nm	35-39 nm	35-39 nm	35-37 nm	27 nm
Receptor	HBGA, Heparan sulphate	Jam-1, Sialic acid	Sialic acid, glycoprotein	HBGA	F-Pilus on *E. coli*
Genome	(+) ssRNA 7.5 kb	(+) ssRNA 7.5 kb	(+) ssRNA 7.5 kb	(+) ssRNA 6.7 kb	(+) ssRNA 3.5 kb
ORFs	3	3	4 (b)	3	-

2 OBJECTIVES

The main objectives of the research described in this thesis were to advance the molecular methods that allow for differentiation between infectious and non-infectious virus particles as well as to improve the reduction in infectious viruses in Pacific oysters.

To accomplish this, three objectives were set as follows:

1 Reduction of false-positive signals in RT-qPCR from inactivated surrogate virus by utilizing pre-treatments in combination with different PCR approaches (Paper I).

2 Monitoring temperature development in oyster tissue and the reduction in virus infectivity in contaminated oysters after selected heat treatments (Paper II).

3 Modifications in the commercial depuration process and their efficacy in eliminating infectious virus from Pacific oysters (Papers III, IV).

3 METHODOLOGICAL APPROACH

Detailed information on chemicals, kits, primers, and probes used in this project is listed in the appendix. Materials and methods are described in detail in Papers I-IV.

3.1 VIRUS CULTIVATION & QUANTIFICATION

TuV was chosen as a cultivable NoV surrogate. TuV seems to be similar to NoV with regard to genome and capsid structure as well as in its binding capability to HBGAs, unlike other common surrogates (see 1.4.3). TuV strain M033 was provided by T. Farkas, Louisiana State University at Baton Rouge, LA, United States. LLC-MK2 cells (ATCC CCL-7) were grown in cell culture flasks in medium M199 with foetal bovine serum and antibiotic/antimycotic solution (complete growth medium). Cells were infected with TuV in medium without additional supplements (maintenance medium). When CPE was observed, supernatant was collected and TuV was harvested by freeze-thawing of the cells. Debris was removed by centrifugation, and the titre of virus stock determined by $TCID_{50}$ or RT-ddPCR.

For $TCID_{50}$ quantification, LLC-MK2 cells were seeded into 96-well plates, grown to confluency, and were inoculated with virus dilutions in quadruplicate. Plates were incubated and checked for CPE daily for up to a week. CPE was converted to $TCID_{50}$/mL by the Spearman and Kärber method described by Hierholzer and Killington (1996).

NoV GII.2 faecal samples were acquired from a patient with acute gastroenteritis and quantified with RT-ddPCR (Persson et al., 2018). GII genotype was determined via the Norovirus Automated Genotyping Tool (Kroneman et al., 2011).

3.2 OYSTERS

Pacific oysters (*Crassostrea gigas*) were obtained from a wild population on the South-Eastern Norwegian coast near Nøtterøy, Vestfold and Telemark County, and were supplied by Norwegian Shores AS, a commercial distributor of Pacific oysters. Oysters were transported chilled to the laboratory and kept at 4°C until experiments were conducted (Papers II-IV).

3.3 ARTIFICIAL CONTAMINATION OF OYSTERS

Bioaccumulation was conducted on a small-scale to evaluate the effect of chlorine on TuV infectivity (Paper IV) and in a larger scale for the depuration experiment on NoV and TuV (Paper III). In the small-scale set up, bioaccumulation was performed for each oyster individually. Each container was covered with a lid and the water was constantly aerated. Large-scale bioaccumulation was conducted at a commercial depuration facility with TuV and NoV simultaneously. After bioaccumulation under constant aeration, oysters were rinsed, and analysed for initial virus concentration. For heat treatment of contaminated oysters (Paper II), oyster DT was spiked with equal volumes of TuV and NoV as insufficient volumes of NoV were available to perform another bioaccumulation.

3.4 DEPURATION

Bioaccumulated oysters were placed in two depuration tanks that differed in water temperature (12/17°C) to evaluate whether elevated water temperatures during depuration facilitate the removal of infectious viruses (Paper III). Depuration lasted four weeks in a flow-through system without water recirculation. Incoming water was disinfected by UV irradiation. Samples were taken weekly from each depuration tank for virus analyses.

3.5 OYSTER PROCESSING & VIRUS RECOVERY

Oyster DT was dissected, homogenised and tissue from several oysters was pooled (three to five oysters). Depending on experimental set up, virus was recovered according to ISO-15216-1 (ISO, 2017) or as described for Hanks' Balanced Salt solution (HBSS) extraction by Araud et al. (2016) with minor modifications. The HBSS method allowed for subsequent quantification of infectious TuV by $TCID_{50}$ in addition to RT-qPCR analysis. For ISO processing, proteinase K was added to homogenised oyster DT and samples incubated at 37°C and 60°C. For HBSS, PBS was added to chopped oyster DT and the tissue was further homogenised with a pestle. Samples were centrifuged and the supernatant was collected for analyses.

3.6 INACTIVATION CONDITIONS

3.6.1 Virus in Medium/Buffer

Virus dilutions were subjected to inactivation conditions (heat, UV and chlorine; Figure 7) to evaluate the different analytical approaches for their efficacy to predominantly detect infectious virus (Paper I). For thermal inactivation TuV aliquots were placed in a heat block for selected time-temperature conditions. For UV light inactivation, virus stock was given into a Petri dish and placed in a sterile bench directly below the UV light source. Aliquots of TuV in growth medium or diluted in PBS were exposed to chlorine for one hour (Paper I, IV). After incubation, an equal concentration of sodium thiosulfate was added.

3.6.2 Oyster Tissue

Artificially contaminated and uncontaminated oysters were subjected to heat treatments to determine how temperature develops within oyster tissue and to assess if any of those treatments may result in a safe product (Paper II). To evaluate the effect of selected heat treatments on virus infectivity, spiked oysters were broiled in a conventional oven or heated on two types of barbecues. Oyster half-shells were placed on a disposable barbecue, on a closed-hood gas grill or into a pre-heated oven set to the grill heater.

In addition, temperature development in oyster DT was monitored during common cooking regimes (steaming, baking/broiling, barbecuing/grilling, boiling water, broth). Oysters were steamed in a steamer basket in a pot of boiling water without touching the water surface. For baking and broiling in the oven, oysters were shucked and placed on a

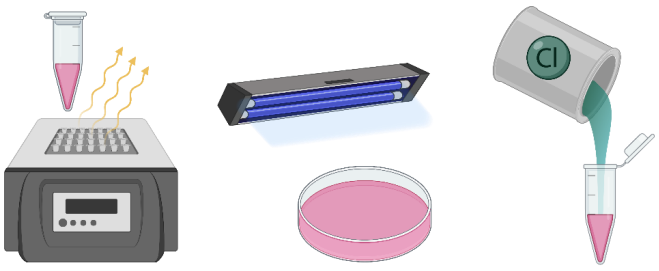

Figure 7: Inactivation treatments TuV was subjected to for the first part of this project (Paper I). Figure created with BioRender (2017).

baking sheet. For broiling, the oven was set to the grill heater. For baking, top and bottom heat were chosen. Shucked oysters were also placed on a small disposable charcoal barbecue or on a gas grill. To monitor temperature development in boiling water, oysters were shucked, and the soft tissue was lowered into the water. For broth treatment, the oyster soft tissue was placed into porcelain cups and simmering broth was added to the cups.

In addition, the effect of a chlorine treatment on TuV in oyster tissue was evaluated to explore a modification that may enhance commercial depuration (Paper IV). Bioaccumulated Pacific oysters were placed into two sea water tanks. After acclimatisation, chlorine was added to one of the tanks for a one-hour incubation. The second tank served as control.

3.7 MOLECULAR ANALYSES

3.7.1 Pre-Treatments

Pre-treatments focused on assessing capsid integrity. Amplification of viruses with damaged capsids in qPCR should be reduced, and thus, false-positive results should decrease. RNase and PMAxx can only access the viral genome after severe capsid disruptions. In that case, RNase will degrade the genome, while the PMAxx dye will bind to RNA and inhibit PCR amplification (Figure 8).

For RNase treatment, RNase A was added to the sample for incubation at 37°C. For PMAxx treatment, samples were incubated at room temperature in the dark before exposure to LED light.

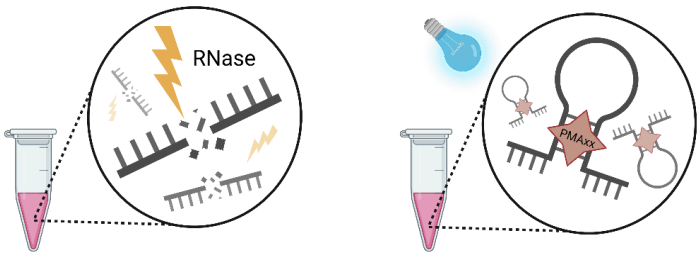

Figure 8: Modifications on viral RNA due to the selected pre-treatments, RNase and PMAxx. Figure created with BioRender (2017).

3.7.1 RNA-Extraction

RNA from virus in medium or buffer was isolated with the QiAmp Viral RNA Kit according to the instruction manual (Paper I). From oyster samples, RNA was extracted with NucliSens magnetic extraction reagents and a MiniMAG device as described in ISO 15216-1 (Papers II, III). Briefly, virus extract was added to lysis buffer and samples were incubated at room temperature before magnetic silica were added. Samples were centrifuged, and the pellet resuspended in a wash solution. Samples were placed in the MiniMAG and washed with three buffers. For elution, samples were incubated in a thermomixer.

3.7.2 Variants of RT-PCR

RT-qPCR

To be able to assess genome integrity, long-range approaches were applied in addition to regular RT-qPCR (Paper I). In RT-qPCR, short ~100 kb amplicons are obtained, which can happen even if other genomic regions are damaged and can thereby lead to false-positive results. In long-range variants, the target sequence is extended. Therefore, genomic damage is more likely to fall within the target region and ideally, no amplicon should be generated. False-positive signals should thus be reduced.

Accordingly, samples were first analysed by one-step RT-qPCR (RTq) in which a short cDNA was generated from the RNA template and then amplified (Figure 9). Next, a long-range nested PCR was designed (Lo PCR). Starting with the same RNA template, a ~2.1 kb cDNA strand was created and amplified in one-step RT-PCR. The PCR product was cleaned and used as a template in qPCR. A two-step long-range RT-qPCR (Lo RT) was designed as well. It is different from RTq in that a long, at least 1.5 kb, cDNA strand was generated prior to qPCR and the reaction was a two-step reaction. For qPCR in both long-range analyses, the same target as in RTq was used. The three PCRs were only applied in Paper I. For the experimental work on oysters a (PMAxx-) RT-qPCR was chosen (Papers II-IV) as long-range PCRs were of greater benefit for UV-inactivated samples, which was not of relevance for those experiments. For the depuration experiment, both viruses were analysed individually, whereas for the heat experiment, a duplex was chosen.

Droplet Digital RT-PCR (RT-ddPCR)

NoV and TuV positive controls were analysed in RT-ddPCR for relative quantification of the depuration samples (Paper III). NoV was analysed according to the method described by Persson et al. (2018), and the TuV assay was developed for this project. The One-Step Advanced Kit for Probes and the QX200 Droplet Digital System were used, and data analysis was performed with the QuantaSoft software.

Primers & Probes

COG2R/QNIF2d primers and QNISP probe were used for NoV GII (Kageyama et al., 2003; Loisy et al., 2005) and the target sequence was 88 bp. For TuV RTq and qPCRs, TVIF primers and probe (Drouaz et al., 2015) were used and resulted in a 107 bp amplicon (Figure 10). Lo PCR was run with TV_LR primers designed in primer BLAST. The resulting PCR product was ~2.1 kb. The RT reaction in Lo RT was primed by the TV_LR reverse primer. The resulting cDNA needed to be ~1.5 kb for TVIF forward primer to bind in qPCR.

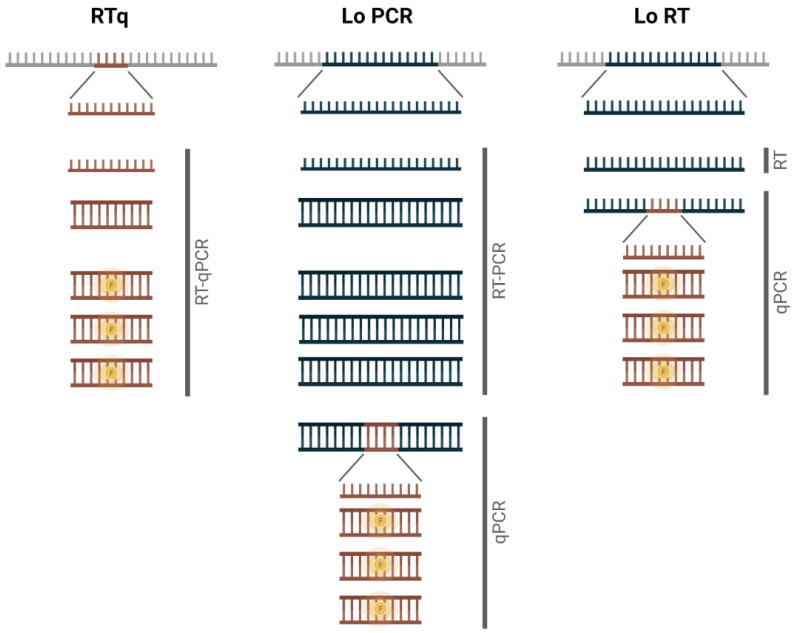

Figure 9: The three molecular approaches. A one-step RT-qPCR (RTq) was run with a ~100 bp amplicon (—). In a long-range RT-PCR (Lo PCR), a ~2 kb target was amplified and used as template in qPCR. In a two-step RT-qPCR (Lo RT), TV_LR reverse primer was used for the RT reaction before cDNA amplification in qPCR. Figure created with BioRender (2017).

Data Analysis

For quantification of genome copy number reductions (Paper I), non-detects (NAs) were set to a Ct value of 40. Thus, reductions given for NA samples are minimum reductions and may be higher in reality. RT-qPCR and qPCR efficiency were determined using standard curves from ten-fold dilution series of stock virus RNA. Relative viral RNA copy numbers were estimated with the following formula:

$$N_1 = N_2 \times (1 + E)^{(Ct_2 - Ct_1)} \quad (1)$$

N_1 and N_2 are the amount of viral RNA in the sample and in the control, respectively; Ct_1 and Ct_2 are threshold cycles for sample and control, respectively; E is the efficiency of amplification.

These numbers were the basis for calculating $\log_{10}$ reductions as follows:

$$log_{10}\, reduction = log_{10}\,(control\ virus) - log_{10}(inactivated\ virus) \quad (2)$$

Statistical analyses were conducted in RStudio and p values <0.05 were deemed significant (R Core Team, 2018; RStudio Team, 2020).

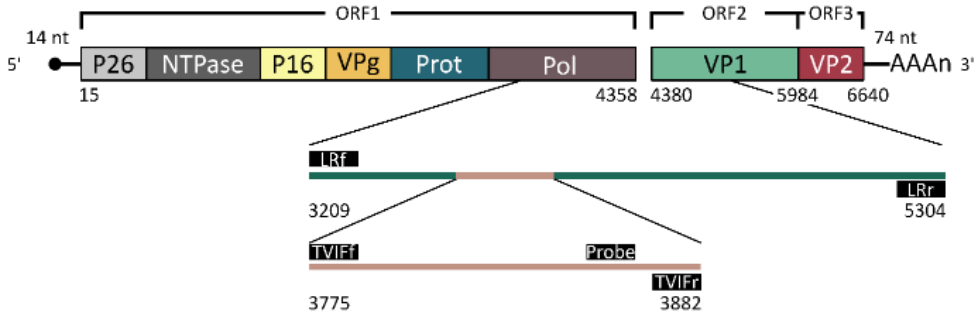

Figure 10: Localisation of primers on the TuV genome. The LR product ranged from base 3,209 to 5,304; the TVIF product ranged from base 3,775 to 3,882. Genome adapted from Farkas et al. (2008).

4 RESULTS & DISCUSSION

4.1 MOLECULAR METHODS TO ASSESS REDUCTIONS IN INFECTIOUS TUV

The most promising approach in NoV cultivation is the replication of the virus in HIEs (Ettayebi et al., 2016) and attempts have been made to cultivate NoV in cell lines. Still, to date, no cell culture routine assay has been established and RT-qPCR remains state of the art for NoV detection, although it does not provide information on virus infectivity. RT-qPCR modifications to identify infectious viruses have been explored, which aim to detect virus capsid or genome integrity. In Paper I, RNase or PMAxx were applied prior to RNA isolation (Figure 8). Pre-treatments should reduce false-positive signals from viruses with damaged capsids. To reduce the detection of viruses with damaged genomes, two variations of a long-range PCR have been applied in addition to standard RT-qPCR (Figure 9). Degradation of the viral genome should reduce the amount of virus detected by the long-range methods (Lo PCR, Lo RT). Combining one of the long-range approaches with a pre-treatment should largely exclude viruses with damaged capsids or genomes from being detected in qPCR. Log_{10} reductions in TuV detected by those molecular approaches were compared to reductions measured by $TCID_{50}$. To evaluate the efficacy of these approaches in determining the loss of virus infectivity, TuV was subjected to inactivation treatments.

To assess which of the three molecular methods mirrors $TCID_{50}$ reductions in TuV after thermal exposure, virus in growth medium was incubated at 56 or 70°C for 5 min or at 72°C for 20 min (Paper I). After thermal exposure, pre-treatments were more advantageous than long-range PCR. On average, RTq with any pre-treatment most closely followed the reduction pattern observed in cell culture (Figure 11). A benefit of pre-treatments is plausible as the capsid is mainly affected by elevated temperatures (Hirneisen et al., 2010). If the capsid is severely disrupted, applied pre-treatments can easily enter the virus and act on the genome. The beneficial effect of these pre-treatments has previously been demonstrated for heat exposed viruses (Lee et al., 2015; Parshionikar et al., 2010; Pecson et al., 2009; Randazzo et al., 2018; Razafimahefa et al., 2021), which highlights the importance of assessing capsid damage after exposure to elevated temperatures. We also observed a benefit of Lo PCR without pre-treatment at 70°C. An advantage of a long-range PCR after heat exposure has been reported previously by Xu et al. (2015). Consequently,

genomic damage may be assessed at increasing temperatures. Genome damage is also induced by UV light. Even so, after we exposed TuV to different UV doses, pre-treatments were beneficial for RTq analysis at the highest UV dose (Figure 11). This may be due to capsid damage at such a harsh UV condition (De Sena and Jarvis, 1981; Hirneisen et al., 2010; Smirnov et al., 1983).

When oysters were spiked with NoV and TuV prior to selected cooking regimes to evaluate virus inactivation in oyster tissue (Paper II), we also observed an advantage of PMAxx. Elevated temperatures encountered during cooking may lead to the disruption of virus capsids and facilitate the entry of the intercalating dye. Accordingly, the effect of PMAxx should be smaller at lower temperatures that affect the capsid less severely. This may explain why PMAxx did not have any advantage for samples on the disposable barbecue but worked well for gas grilled and broiled samples. Only cell binding capacity may have been affected on the disposable barbecue, which would explain the observed reduction in TuV infectivity. In addition, we found no effect of PMAxx on TuV or NoV samples taken during the depuration trial (Paper III). In contrast to the positive effect of PMAxx for heat treated viruses (Papers I, II), the capsid was probably not exposed to conditions harsh enough to induce severe damage during depuration, similar to samples on the disposable barbecue.

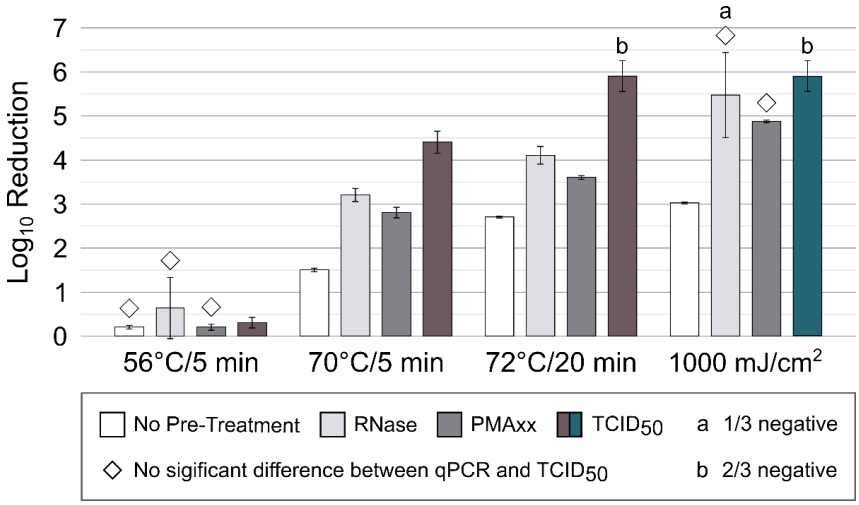

Figure 11: Log_{10} reduction in TuV after heat and harsh UV treatments as detected by one-step RT-qPCR = RTq. Log_{10} reductions are depicted for $TCID_{50}$ (■/■) and RTq analyses without pre-treatment (□), with RNase (■), and PMAxx (■). ◇ No statistically significant difference between RTq and $TCID_{50}$; (a) 1/3, and (b) 2/3 samples are negative; error bars represent standard errors.

4.1.1 Methodological Considerations of Long-Range Analyses

While thermal treatment initially acts on the viral capsid, the primary target of UV light is the genome. UV exposure leads to the formation of pyrimidine dimers, which inhibit amplification (Hirneisen et al., 2010; Miller and Plagemann, 1974). This type of damage may go unnoticed if the PCR target is around 100 bp. In a long-range PCR, with an extended amplicon length, dimer formation is more likely to inhibit amplification. This explains the advantage of Lo PCR observed in Paper I when UV treated samples were analysed (Figure 12a), while no clear benefit of pre-treatments could be detected. Previous findings support the advantage of long-range analysis for viruses exposed to UV light (Ho et al., 2016; Rodriguez et al., 2013; Simonet and Gantzer, 2006; Wolf et al., 2009). Exposure to the harshest UV dose (1000 mJ/cm^2) resulted in the near complete loss of TuV infectivity (~5.9 $\log_{10}$ reduction). At this inactivation level, no genomic material was detected with Lo PCR. Presumably, Lo PCR NAs represent a reduction in TuV below the limit of detection after the harsh condition. This would suggest Lo PCR without a pre-treatment is among the methods that closely resembles $TCID_{50}$ results after UV exposure.

Despite the observed advantage of Lo PCR in assessing genomic damage, Lo PCR was not a reliable assay, especially in combination with RNase (Figure 12b). In addition, Lo PCR became more unpredictable with harsher inactivation conditions. This may be connected to the high sensitivity for genomic damage since Lo PCR has the longest target sequence (~2.1 kb) of the three molecular methods. No TuV RNA was detected with RNase Lo PCR except for at the mildest heat and UV conditions. Overall, high Ct values, NAs, and the largest standard errors were observed for most Lo PCR analyses with RNase. This was also the case for some samples that were not exposed to any of the inactivation conditions and occasionally for RTq and Lo RT. Possibly, RNase was still active after the pre-treatment despite cooling the samples on ice. Still, adding lysis buffer with guanidine thiocyanate should have led to RNase inactivation. Also, preliminary testing did not find any difference in Ct values when RNase inhibitor was added after RNase exposure (data not shown). Still, RNase may be an unsuitable candidate for the assessment of capsid damage of RNA viruses since enzyme activity needs to be abolished to not negatively interfere with downstream applications.

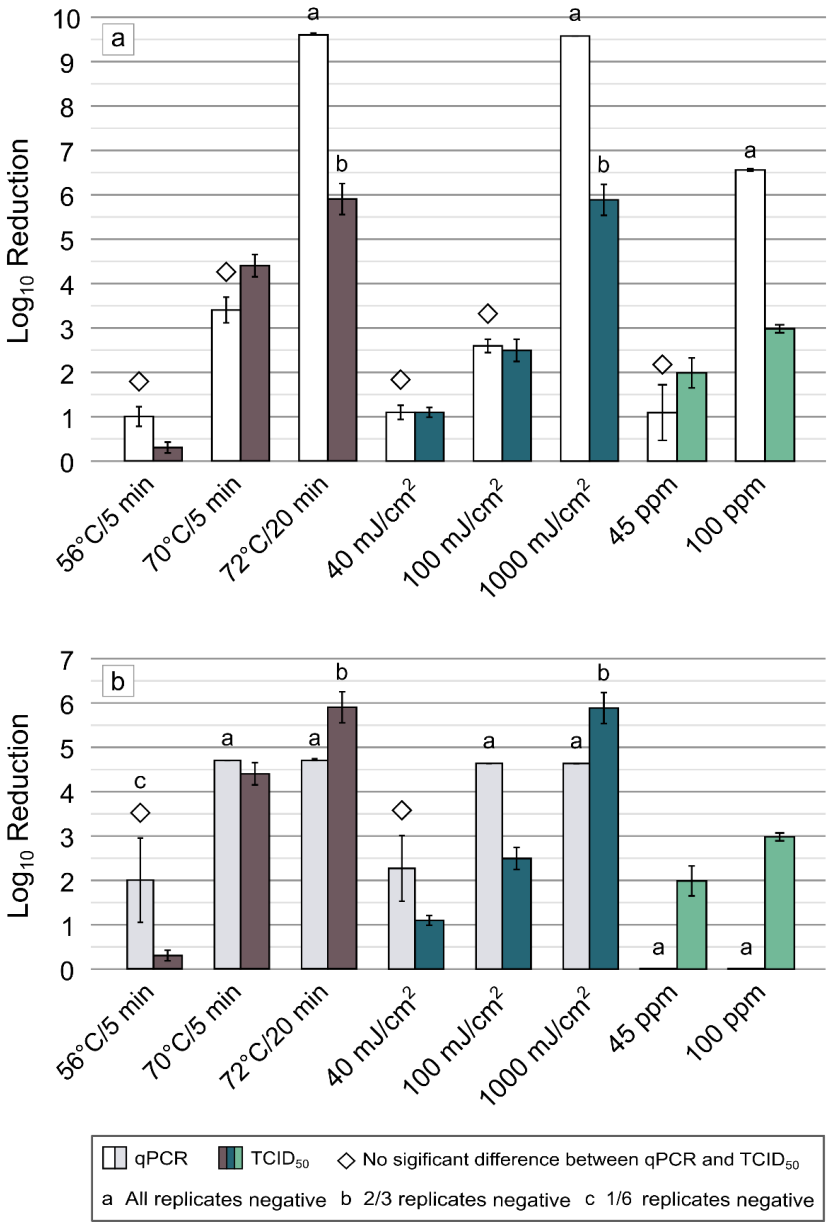

Figure 12: Log_{10} reduction in TuV after exposure to inactivation treatments as detected by long-range RT-PCR + qPCR = Lo PCR. Log_{10} reductions are depicted for $TCID_{50}$ (■/■/■) and Lo PCR without pre-treatment (12a, □) and with RNase (12b, ■); No log_{10} reductions are depicted for 45 and 100 ppm with RNase as both the control and the chlorine-treated samples were negative in qPCR. ◇ No statistically significant difference between Lo PCR and $TCID_{50}$; (a) All replicates negative; (b) 2/3 replicates negative; (c) 1/6 replicates negative; error bars represent standard errors.

The two long-range methods, Lo PCR and Lo RT, differed to a greater extent from one another than expected (Paper I). This difference may be a consequence of amplicon length. In Lo PCR, the RT-PCR product is ~2.1 kb, while in Lo RT, the generated cDNA only needs to be ~1.5 kb to enable qPCR amplification with TVIF primers (Figure 10). Accordingly, Lo PCR is more sensitive for genomic damage and virus reductions are higher with this method compared to Lo RT after exposure to UV light and chlorine (Table 2).

Overall, utilising pre-treatments and long-range PCRs can offer an opportunity to reduce false-positive results from inactivated viruses. The suitability of each method for correctly assessing virus reduction depends on mode of inactivation. A reliable method that performs equally well for all modes of inactivation would be challenging to design. Moreover, some methods applied in Paper I contain multiple steps, and are not well-suited for routine analysis. Cross-contamination and accumulation of errors are possible as observed for Lo PCR. For instance, the Lo PCR product was cleaned to remove short nucleic acids prior to qPCR. During this process, the amount of PCR product retained by the column may have differed and could have contributed to the unstable results we observed after qPCR analysis.

As the application of the long-range methods caused some challenges, and extensive genomic damage was not expected in further experiments, we chose not to develop the long-range analyses any further. For the analysis of oyster samples, RT-qPCR was chosen. To assess capsid damage, which was expected to occur at least after heat treatments in Paper II, a pre-treatment was included. PMAxx was selected for the remainder of the project since RNase may be more unreliable, as discussed above.

Table 2: Comparison of $\log_{10}$ reductions in TuV infectivity ($TCID_{50}$) and TuV RNA (Lo PCR, Lo RT) after UV and chlorine treatments in Paper I. ◇ No statistically significant difference between qPCR and $TCID_{50}$; (a) No replicates detected in qPCR.

Treatment	Reduction $TCID_{50}$	Reduction Lo PCR	Reduction Lo RT
40 mJ/cm²	1.1 $\log_{10}$	1.1 $\log_{10}$ ◇	0.3 $\log_{10}$
100 mJ/cm²	2.6 $\log_{10}$	2.6 $\log_{10}$ ◇	0.5 $\log_{10}$
1000 mJ/cm²	5.9 $\log_{10}$	9.6 $\log_{10}$ (a)	2.2 $\log_{10}$
45 ppm	2.0 $\log_{10}$	1.1 $\log_{10}$ ◇	0.5 $\log_{10}$
100 ppm	3.0 $\log_{10}$	6.6 $\log_{10}$ (a)	1.8 $\log_{10}$

4.2 VIRUS INACTIVATION DIFFERS DEPENDING ON THERMAL PROCEDURE

Heat inactivation resulted in significant reductions in TuV in growth medium (Paper I). After exposure to three time-temperature combinations, reductions of 0.3, 4.4 and 5.9 $\log_{10}$ were observed. Enteric viruses should therefore lose infectivity during sufficient thermal treatment. Oysters, especially consumed raw, are linked to NoV gastroenteritis and bacterial illness. So, the United States Centers for Disease Control and Prevention (CDC) recommend a heat treatment to obtain a safer product (CDC, 2019a; b). To prevent NoV infections, oysters should not be consumed if they measure <60°C (CDC, 2019a). According to published data, the temperature should be higher to inactivate human pathogenic viruses in shellfish. Most studies indicate temperatures >85°C should be maintained for several minutes (see 1.3.3). To gain insight into which temperatures are reached in oysters during commonly applied and CDC-recommended cooking procedures, temperature development in oyster DT was monitored (Paper II). Thermal treatments included steaming, broiling/baking in the oven, grilling (disposable charcoal barbecue, gas grill), boiling water and broth that was poured over oyster soft parts. Recorded temperatures were evaluated for their potential to inactivate NoV. In addition, oysters were spiked with NoV and TuV prior to selected cooking regimes (broiling, disposable barbecue, gas grill) to evaluate virus inactivation in oyster tissue. Virus recovery was performed according to the ISO standard (ISO, 2017) and as described for HBSS extraction in Araud et al. (2016) to be able to conduct cell culture analyses. Samples were analysed before and after cooking treatments by $TCID_{50}$ (TuV) and (PMAxx-) RT-qPCR, as we found RTq with pre-treatments most closely resembled results from $TCID_{50}$ after heat exposure (Paper I).

When temperature in oyster DT was monitored, temperature development within oysters differed depending on preparation method. Results of the thermal treatments are compiled in Table 3 and Figure 13. The lowest temperatures were observed in broth (<60°C, ●) and for some oysters on the disposable barbecue (<50°C, ◆). This would not result in a safe product in terms of NoV as illustrated when insufficient heating of clams led to a NoV outbreak (Lunestad et al., 2016). Temperatures rose most quickly in boiling water (◆), reaching 80°C after 3 min. Steamed oysters (●) reached the same temperature after 5 min and opened after 6 min at >90°C. Accordingly, applying those methods should result in a safe product (Flannery et al., 2014; Pilotto et al., 2019).

Table 3: Applied thermal treatments and CDC recommendations for oyster preparation (CDC, 2019b); * no CDC recommendation available, recipe suggestions were used instead (Burnett, 2020; Lunestad et al., 2016; Mortensen et al., 2017b). The CDC's recommended temperature for shellfish consumption to reduce the risk of NoV infection is listed (CDC, 2019a), as well as the average temperatures measured after the recommended cooking time. Reductions in infectious TuV as measured in cell culture ($TCID_{50}$) are included for selected treatments. ND = Not determined.

Thermal treatment	Recommended treatment and shellfish temperature	Measured temperature after recommended time	CDC-temperature reached	Comments	$TCID_{50}$ Reduction (TuV)
Steaming	4-9 min - 60°C	66.3 ± 9.9°C (4 min) 98.6 ± 0.8°C (8 min)	Yes	Shell opening: 92.4 ± 3.3°C (6 min)	ND
Broiling	3 min, 7.5 cm from heat - 60°C	52.0 ± 19.3°C	No	Ready: 78.0 ± 12.3 (5-6 min)	3.6 log_{10} 73.4 ± 13.8°C (5 min)
Baking	10 min at 232°C - 60°C	73.0 ± 10.8°C at 225°C	Yes	Ready: 48.8 ± 10.3° C (5 min)	ND
Barbecue (Disposable)	* Edges curl, liquid bubbles - 60°C	48.9 ± 16.6°C (6 min)	No	~35°C difference depending on placement after 8 min	1.2 log_{10} 55.9 ± 19.2°C (8 min)
Barbecue (Gas)	* 2-6 min with closed lid - 60°C	38.8 ± 9.4°C (2 min) 82.0 ± 10.7°C (6 min)	Yes (≥4 min)	-	3.1 log_{10} 74.5 ± 11.3°C (5 min)
Boiling (Shucked)	≥3 min - 60°C	80.3 ± 1.0°C (3 min)	Yes	-	ND
Broth	* Add just before serving - 60°C	<60°C (5 min)	No	Confirmed outbreak at the same conditions	ND

In the oven, temperature development depended on the placement of oysters in relation to the heat source (Figure 13). With top and bottom heat (baking, ◆), temperature rose more slowly than with the grill function (broiling, ●). This may be due to the oyster shell insulating against heat from below and the greater distance from the heat source at the top of the oven during baking. When baked oysters were ready after 5 min, oyster temperature (~49°C) would not suffice for significant virus reductions. Extending baking times to 10 min (~73°C) might not suffice either (Croci et al., 2012; Lunestad et al., 2016; Shao et al., 2018; Sow et al., 2011). Nevertheless, we demonstrated a significant reduction (3.6 $\log_{10}$) in infectious TuV in broiled oysters that measured ~73°C after 5 min. This resembled almost a complete loss of TuV infectivity and implies oysters may safe after broiling.

The type of barbecue influenced heat development in oyster DT (Figure 13). On the disposable charcoal barbecue, placement of the oysters was crucial. When oysters were considered ready after 6 min, they had reached ~68 and ~37°C above well (●) or poorly (◆) glowing charcoal, respectively. Higher temperatures were recorded on the gas grill (◆). TuV infectivity showed a greater reduction after 5 min on the gas grill compared to 8 min on the disposable barbecue (3.1 and 1.2 $\log_{10}$, respectively), which is in accordance with the higher temperatures on the former. Still, infectivity was not completely abolished on the gas grill, at ~75°C. Again, the effect of the oyster shell insulating against heat from below may be considered. Previous findings suggest temperatures on either barbecue may not guarantee food safety, and outbreaks have been linked to grilled oysters (Araud et al., 2016; Croci et al., 2012; McDonnell et al., 1997; Shao et al., 2018; Sow et al., 2011).

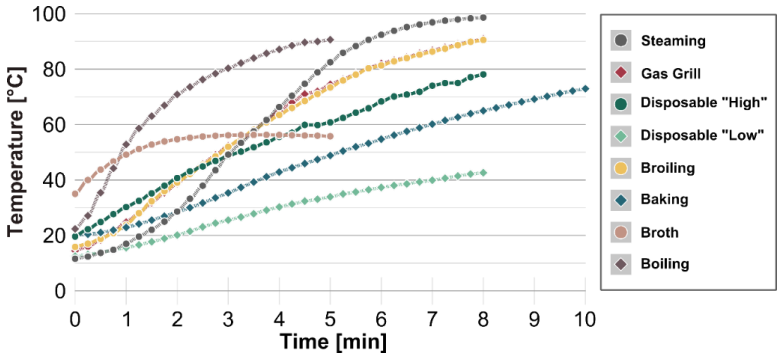

Figure 13: Temperature development in Pacific oyster DT over time while steaming (●), baking (◆) and broiling (●) in the oven, on the disposable barbecue, divided into "low temperature" (◆) and "high temperature" (●), on a closed gas grill (◆), in boiling water (◆) and in broth (●).

4.2.1 Effect of Virus Recovery Method & Data Interpretation

RT-qPCR underestimated the reduction in infectious TuV compared to $TCID_{50}$ (Figure 14). A PMAxx pre-treatment resembled $TCID_{50}$ reductions more closely, as mentioned in 4.1. The effect of PMAxx was also greater when heat-treated samples were extracted with the HBSS method. The amount of RNA detected after HBSS extraction with PMAxx was comparable to what was detected after ISO extraction without PMAxx. This may be linked to the proteinase K incubation at 60°C in the ISO protocol (ISO, 2017). Capsids that have already been affected by the oyster cooking treatment may have been additionally damaged during this step (Langlet et al., 2018). For the assessment of virus reductions after thermal exposure HBSS with PMAxx may be an alternative to avoid a possible influence of the proteinase K treatment on the detection of infectious virus.

It was also observed that the amount of NoV RNA increased after oyster cooking, primarily on the disposable barbecue (Figure 14). The same was observed in a previous setup (data not shown) and occurred independently of the person performing the virus recovery and sample analysis. Possibly, elevated temperatures facilitate NoV recovery from oysters. NoV may have been more easily released from the heat-treated oyster than from the raw control. Temperatures would still have been too low to disrupt the capsid on the disposable barbecue. Thus, an overall increase in NoV RNA would be detected. At higher temperatures on the gas grill and during broiling, virus recovery would still be positively affected, but due to capsid and possibly genome damage, NoV RNA decreased.

Several publications indicate that oyster tissue has a protective effect against elevated temperatures. Tougher time-temperature combinations are required to inactivate virus in shellfish compared to virus in buffer solutions (Araud et al., 2016; Croci et al., 2012; Park et al., 2015). After exposure to 70°C for 10 min, MNV was reduced by 3.0 log_{10} in abalone shell, whereas in cell culture lysate MNV decreased by 4.5 log_{10} (Park et al., 2015). When FCV was exposed to 80°C for 3 min, infectious FCV in suspension was reduced by 4.5 log_{10}. In mussels, FCV was only reduced by 2.2 log_{10} (Croci et al., 2012). The same study showed that the reduction in NoV RNA was 2.6 log_{10} smaller in mussels compared to in suspension. We also observed a larger reduction in infectious TuV at 70-75°C in medium than in oysters. An average 3.4 log_{10} reduction was observed after grilling and broiling when

oyster DT was ~74°C after 5 min. The reduction in medium at 70°C/5 min was ~1.0 $\log_{10}$ larger (Table 4). This suggests TuV was better protected within the oyster DT than in medium. Still, during the two cooking procedures the in-tissue temperature was not >70°C throughout the whole experiment (Figure 13), and reductions may have been higher if that temperature would have been held for the whole experimental time. The 200 µL samples of virus in medium would have reached the target temperature more quickly than the oyster tissue during cooking. Thus, the protective effect may simply stem from the additional time it takes for the oyster tissue to reach a specific temperature. Moreover, sometimes the reduction in infectious TuV was smaller in medium. For example, on the disposable barbecue the oysters reached a temperature of 56°C on average, and reductions were 0.9 $\log_{10}$ larger than in medium at the same temperature. One explanation for this might be the 3 min longer exposure time. However, this may not be the sole reason, as on the barbecue a temperature >50°C was only held for 1 min 45 s. Furthermore, the temperature varied across the barbecue so that the placement of oysters was crucial. Oyster temperature varied greatly on the disposable barbecue (Figure 13). Therefore, the high reductions on the barbecue may be a result of virus reductions in oysters that have reached a high temperature

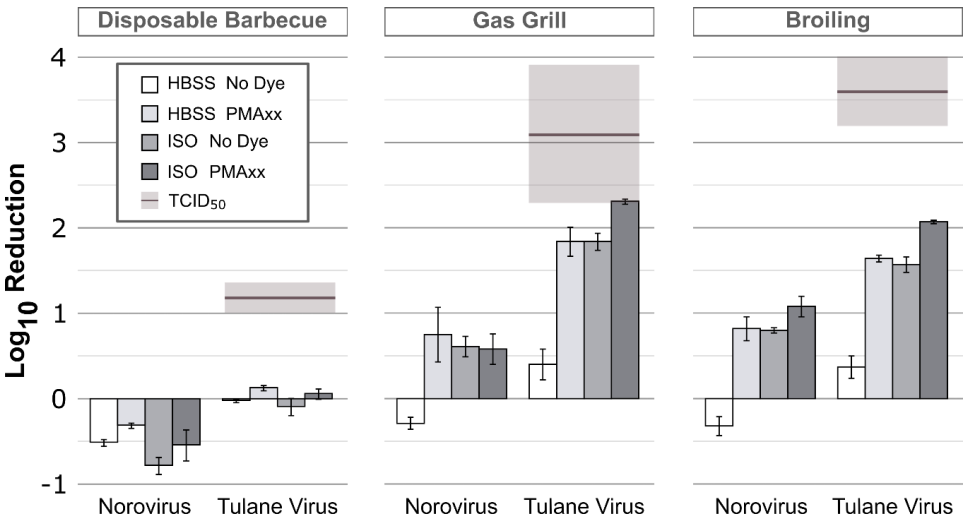

Figure 14: Reductions in NoV and TuV RNA as determined by RT-qPCR with (▫/▪) and without (□/▫) the addition of PMAxx for the two virus extraction methods, HBSS (□/▫) and ISO/Proteinase K (▫/▪). Arithmetic mean of three replicates and standard errors are depicted. For comparison, average $TCID_{50}$ reductions (—) and standard error (▫) are included.

and may have skewed the average reduction. In general, we only observed a slight protective effect of the shellfish tissue. The difference between reductions in medium and oyster at 70-74°C was <1.0 $\log_{10}$. In contrast, publications that report a protective effect found differences between 1.5 and 2.6 $\log_{10}$ (Croci et al., 2012; Park et al., 2015). Virus diluted in buffer is sometimes used for heat experiments (Croci et al., 2012), whereas we have worked with virus in cell culture medium. Proteins and other components of the medium may protect the virus from heat damage, which could explain the discrepancy between our results and other publications. In addition, our study was the only one that simulated practical cooking techniques. Croci et al. (2012) and Park et al. (2015) worked with homogenised tissue that was spiked with virus and exposed to heat in a water bath. Araud et al. (2016) worked with bioaccumulated oysters, but still placed samples in a water bath. This suggests that elevated temperatures affect viruses differently, depending on mode of exposure. Several other studies were conducted that worked with spiked homogenised bivalve tissue (Jeon et al., 2020; Shao et al., 2018; Sow et al., 2011). Virus distribution may vary in spiked oysters. This may result in varying levels of protection. The protective effect may thus be dependent on how oysters were contaminated or the state of bivalve tissue and could be further investigated.

There are some additional limitations regarding the interpretation of related work on virus inactivation. The techniques used for bivalve contamination and heat exposure differ, which makes results not easily comparable between publications. Moreover, different bivalve species are worked with so that recommendations based solely on the comparison of our recorded data to time-temperature combinations reported for virus inactivation in the literature must be critically scrutinised.

Table 4: Comparison of $\log_{10}$ reductions in TuV infectivity after heat treatments in Papers I and II.

Paper I - in medium		**Paper II - in oysters**			
Treatment	**Reduction**	**Treatment**	**Time**	**Temperature**	**Reduction**
56°C/5 min	0.3 $\log_{10}$	Disposable	8 min	56 ± 19°C	1.2 $\log_{10}$
70°C/5 min	4.4 $\log_{10}$	Gas grill	5 min	73 ± 14°C	3.1 $\log_{10}$
72°C/20 min	5.9 $\log_{10}$	Broiling	5 min	75 ± 11°C	3.6 $\log_{10}$

As discussed above, TuV infectivity considerably decreased after the cooking treatments in Paper II. On the contrary, no major reductions in TuV could be detected in an earlier set up (Figure 15). Reductions were only observed when the experiment was repeated. This repetition resulted in the data discussed in the present work. Evidently, there is a discrepancy between these two experiments. Thus, the experiment has to be conducted a third time prior to submitting the manuscript, to verify the data presented in this thesis.

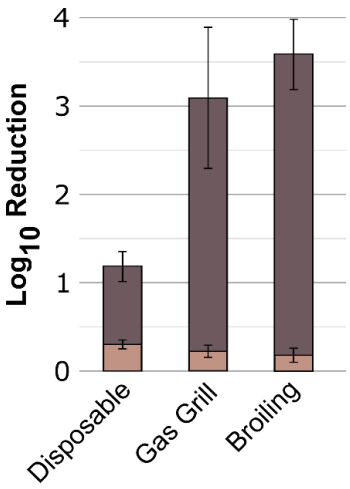

Figure 15: Log_{10} reductions in TuV titre in spiked oysters after exposure to the three heat treatments (disposable barbecue, gas grill, broiling). (■) Reductions July 2021; (■) Reductions November 2021; error bars represent standard errors.

4.3 MODIFICATIONS IN THE DEPURATION PROCESS

As discussed in Paper II, a heat treatment of oysters may be a strategy to reduce the number of infectious enteric viruses. However, we demonstrated that the inactivating effect differs between cooking methods (Table 3). Not all methods are equally suited for virus inactivation and there may also be a difference between cooking equipment. Thus, a universal time-temperature recommendation may be difficult to establish. Also, the CDC recommends the consumption of shellfish of at least 60°C to make sure NoV is inactivated. As elaborated in Paper II, this temperature would most likely not suffice for inactivation. To obtain a safe product, temperature within oyster tissue should be higher, but this often negatively affects taste and consistency. Therefore, it is more desirable to produce oysters that are free of viral contaminants so that a harsh heat treatment would not be necessary. Growing oysters in pristine waters without faecal contamination is a prerequisite for this, but even in class A areas with low levels of faecal indicator bacteria in oyster tissue NoV may be present. Thus, oysters are placed in clean water for depuration or relaying to tentatively eliminate viral pathogens.

The depuration process has been proven to be rather inefficient at removing pathogenic enteric viruses from bivalve molluscs. Even though bacterial pathogens are quickly eliminated, NoV is often still detectable in depurated oysters and can lead to food-borne infections. To improve the efficacy of depuration, numerous improvements have been suggested as reviewed in Martinez-Albores et al. (2020).

4.3.1 Elevated Water Temperatures Facilitate Virus Reduction During Depuration

One proposed strategy to improve depuration efficacy is an increase in water temperature. Up to 28°C, oyster filtration rate, oxygen consumption and enzymatic activity increase (Brock et al., 1986; Hutchinson and Hawkins, 1992; Kim, 1995; Numaguchi, 1994; Sytnik and Zolotnitskiy, 2014). Oyster metabolism is also shown to increase with temperature (Lees et al., 2010). Therefore, the elimination of pathogens may improve at elevated temperatures, and there is evidence that a warmer water temperature facilitates virus removal during depuration (Bachur, 1988; Choi and Kingsley, 2016; Kingsley et al., 2018;

Rupnik et al., 2021; Younger et al., 2020). To assess whether elevated temperatures improve the elimination of NoV and TuV from oyster tissue, both were bioaccumulated in Pacific oysters (Paper III). Contaminated oysters were depurated at 12 and 17°C for 28 days (Figure 16). Infectious TuV as well as TuV and NoV RNA were monitored weekly. Depuration at elevated temperatures should increase the removal of virus particles from oysters.

According to $TCID_{50}$, infectious TuV gradually decreased during the depuration period (Figure 17). After seven days, TuV was reduced by ≤ 0.8 log_{10}. After four weeks, TuV titre had dropped by ≥ 4.2 log_{10}, regardless of water temperature. The difference in TuV reduction between the two water temperatures was significant, especially on days 14 and 21. Reductions on those days were ≥ 1.3 log_{10} higher at 17°C. On day 21, reductions >3.0 log_{10} were observed at 17°C, while at 12°C reductions were still <2.0 log_{10}. Previous findings illustrate the benefit of elevated temperatures during depuration (Bachur, 1988; Kingsley et al., 2018; Neish, 2013).

After our depuration trial we observed minimal reductions of <0.8 log_{10} in TuV RNA (Figure 17). Although it is not uncommon that RT-qPCR underestimates virus reduction compared to cell culture assays, Polo et al. (2018) reported more substantial reductions in

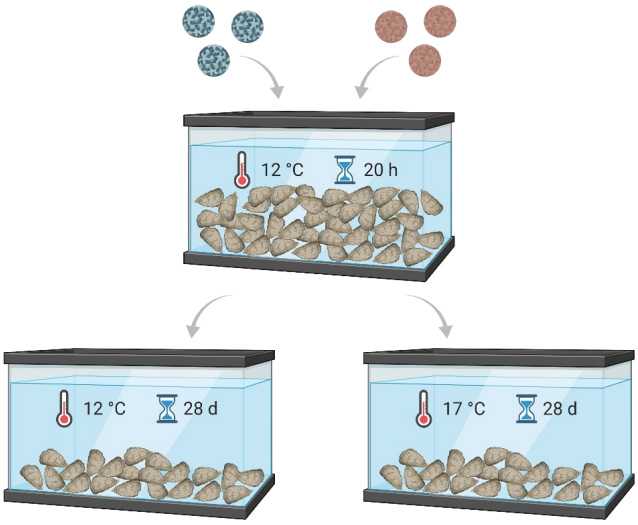

Figure 16: Experimental set up for depuration at elevated temperatures. Bioaccumulation was performed with TuV and NoV simultaneously. Oysters were separated after bioaccumulation and depurated at two water temperatures for 28 days. Figure created with BioRender (2017).

TuV RNA at comparable temperatures. However, they found no difference in TuV RNA persistence during depuration at 17°C and 11°C. Likewise, we observed no statistical advantage of the higher temperature with RT-qPCR.

NoV RNA decreased by 0.8 $\log_{10}$ after seven days (Figure 17). Similar reductions after a one-week depuration have been reported previously (Choi and Kingsley, 2016; Rupnik et al., 2021; Younger et al., 2020). As with TuV RNA, we could not detect a difference in NoV RNA reduction between the two water temperatures. Although Neish (2013) only reports a minor improvement in NoV reduction at elevate water temperatures, a beneficial effect of elevated temperatures on NoV reduction has been repeatedly suggested (Choi and Kingsley, 2016; Rupnik et al., 2021; Younger et al., 2020). Possibly, a greater difference in water temperature results in a greater difference in depuration kinetics (Rupnik et al., 2021). Our experiment was conducted in summer when sea water was ≥11.5°C. This water was used directly for the low temperature tank. Ideally, the depuration trial should be repeated during the winter months with natural water temperatures <10°C for the low temperature depuration.

Curiously, we observed a more rapid decline in NoV RNA compared to TuV during the first week. NoV and TuV were bioaccumulated simultaneously, so that there may have been competition over HBGA receptors, to which both viruses bind. TuV may have outcompeted NoV, either because of its higher concentration or a possibly stronger binding affinity. There is some evidence that GII.2, the genotype we used in the present work, does not bind as well to HBGAs as other NoV genotypes and HBGA-type preference differs among publications (Harrington et al., 2002; Harrington et al., 2004; Huang et al., 2005; Singh et al., 2016; Tenge et al., 2021). In oysters, GII.2 bioaccumulation has been unsuccessful before (Ueki et al., 2021), although *in vitro* tissue binding and oyster-related outbreaks have been reported for the genotype (Iritani et al., 2014; Langlet et al., 2015; Meghnath et al., 2019; Zhang et al., 2021a). Another explanation for the more rapid decline in NoV RNA in the present work could be found in the seasonal variations in NoV binding to oyster tissue as demonstrated by Maalouf et al. (2010) who state that HBGA receptor expression is higher during late winter and spring. We conducted our study in mid-summer, so that HBGA expression in oyster DT may have been decreased. All these factors may

have contributed to the rapid decline in NoV. Most NoV may have been present as an unbound fraction in the oyster DT and would have been eliminated quickly.

In general, we found an advantage of elevated water temperatures, but this was only noticeable after 14 days of depuration when infectious TuV was reduced by 1.0 $\log_{10}$ and 2.6 $\log_{10}$ at the lower and higher temperature, respectively. These results imply elevated temperature may primarily be relevant for prolonged depuration. Commercial depuration is supposed to last ~48 h, depending on region, and is rarely extended to more than five days (Lee et al., 2008; McLeod et al., 2017b). Short depuration may not benefit from raised temperatures, and only facilities that voluntarily extend depuration to several weeks would observe an advantage. After 28 days, we detected reductions in TuV infectivity of ≥4.2 $\log_{10}$ at both temperatures. Thus, the economic burden of heating up the depuration water must be weighed against the burden of prolonging the depuration period. Regardless of water temperature, an extension of the oyster depuration period to more than seven days should be beneficial for the inactivation of viral pathogens.

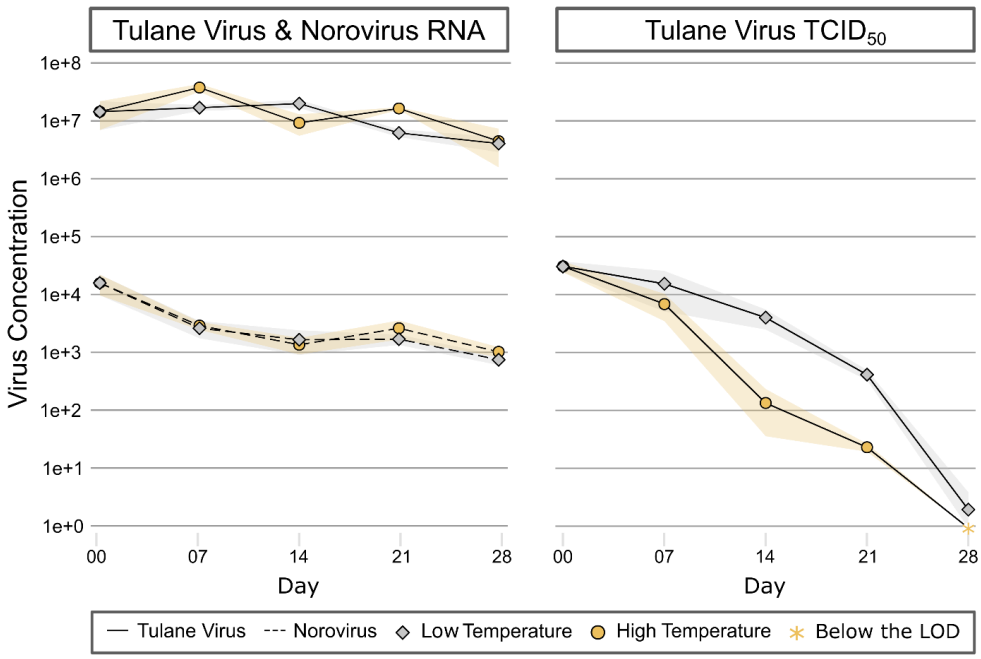

Figure 17: Concentration of TuV (—) and NoV (---) RNA in genome copies per mL as determined by RT-qPCR without the addition of PMAxx, as well as TuV tire in $TCID_{50}$ per mL during the depuration period at high (●) and low (◆) water temperatures (17°C, 12°C); the ribbon represents standard errors of three replicates. ✳ = Below the limit of detection (LOD).

4.3.2 Antiviral Agents Need to Reach the Oyster Digestive Tract at Adequate Concentrations

A prolonged depuration time and elevated water temperatures as in Paper III may be a way to eliminate most infectious NoV from Pacific oysters, but it may not always be feasible. An extension of the depuration period would lead to additional costs, especially if water temperatures are supposed to be >10°C year-round. Therefore, a more economical approach may be preferable by commercial oyster distributers. Since chlorine has already been shown to have an inactivating effect on human enteric viruses in water (Cromeans et al., 2010; Keswick et al., 1985; Kitajima et al., 2010; Peterson et al., 1983), it may also influence the infectivity of viruses in food matrices. In a small-scale pilot, this last part of the project assessed the antiviral effect of chlorine at 45-200 ppm on TuV in cell culture medium and PBS. It was also evaluated whether a one-hour treatment of oysters with chlorine may facilitate the reduction in virus infectivity (Figure 18; Paper IV). TuV had already been exposed to 45 and 100 ppm to evaluate the molecular assays in Paper I.

Reductions due to chlorine treatments were difficult to assess with the molecular methods in Paper I. The mode of virus inactivation by chlorine is not unambiguously determined. Still, it has been shown that both the capsid and the genome are affected (Fuzawa et al., 2019; Hirneisen et al., 2010; Li et al., 2002; O'Brien and Newman, 1979; Wigginton and Kohn, 2012). Accordingly, there should be a benefit of both the pre-treatments and long-

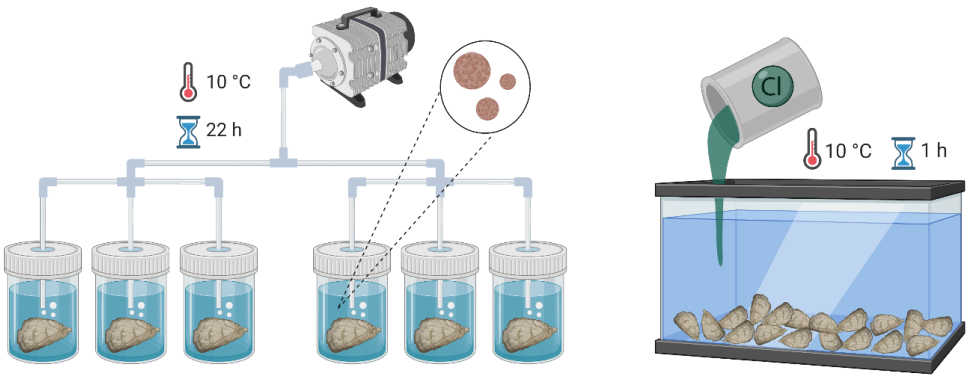

Figure 18: Experimental set up for chlorine treatment of contaminated oysters. Bioaccumulation was performed for each oyster individually. Oysters were collected after bioaccumulation and subjected to 45 ppm chlorine for one hour. Figure created with BioRender (2017).

range PCRs. In this context it is surprising that Lo PCR was least suited to detect reductions after chlorine exposure. Most Lo PCR analyses resulted in NAs. Amplification may have been impossible due to severe genome damage. In contrast, $\log_{10}$ reductions were more correctly estimated by Lo RT, the other molecular analysis that utilized a long target sequence. An additional advantage of pre-treatments could be observed as well (Figure 19). Similarly, an advantage of intercalating dyes and long-range PCR for estimating virus reductions after chlorine exposure has been reported (Fuster et al., 2016; Leifels et al., 2015; McLellan et al., 2016; Xu et al., 2015). However, as with Lo PCR, we observed a high number of NAs in Lo RT, especially at a concentration of 100 ppm. Since none of the tested methods performed especially well for chlorine treated TuV (Paper I), only $TCID_{50}$ was chosen for the subsequent analyses of chlorinated samples (Paper IV).

When TuV was diluted in PBS, chlorine exposure led to concentration-dependent $\log_{10}$ reductions (Figure 20). TuV was reduced by 1.5 to >3.0 $\log_{10}$, with chlorine ranging from 45 to 200 ppm. These concentrations are in line with what has previously been reported to achieve TuV reductions (Hirneisen and Kniel, 2013; Tian et al., 2013; Wang et al., 2014; Xu et al., 2015). In contrast, we observed no significant reduction in virus titre for TuV in growth medium or in oyster DT after exposure to 45 ppm. One explanation for this discrepancy in reductions may lay in the factors that influence chlorine efficacy, for instance organic matter and temperature (Butterfield et al., 1943; Hirneisen et al., 2010; Kelly and Sanderson, 1958; Urakami et al., 2007).

The efficacy of chlorine is higher at elevated temperatures (Kelly and Sanderson, 1958). Accordingly, the 10°C sea water used during the chlorine treatment of oysters in the present study may have led to a reduced effect. Thus, water temperature may be increased in future experiments. On a commercial scale, raising the water temperature may prove unfeasible due to extra costs as mentioned in Paper III. Conversely, several publications report a high chlorine efficacy at 5°C (Cromeans et al., 2010; Lim et al., 2010; Shin and Sobsey, 2008). In these studies, equipment has been made chlorine-demand free (CDF). Chlorine concentrations during the actual experiment will thus not immediately decline and a low experimental chlorine concentration may already lead to substantial reductions in a CDF environment. In contrast, in an environment with high chlorine demand, free chlorine concentrations rapidly decline (Urakami et al., 2007). This effect of organic matter may

account for the lack of inactivation we observed for TuV in medium and oysters. Also, organic material is bound to be present in sea water. Thus, the only possibility is to increase chlorine concentrations, but this would also increase harmful chlorine by-products (Di Cristo et al., 2013; Gil et al., 2019), may give an undesirable chlorine smell to oyster meat (Kjelseth, 2021), and may negatively impact oyster physiology (Galtsoff, 1946; Ren and Su, 2006). The localisation of virus is an additional challenge that needs to be overcome. Chlorine may not retain any activity before it reaches the DT. Microencapsulation may be a solution, but again, the capsule's organic material would reduce chlorine efficacy. Alternatively, different agents should be chosen.

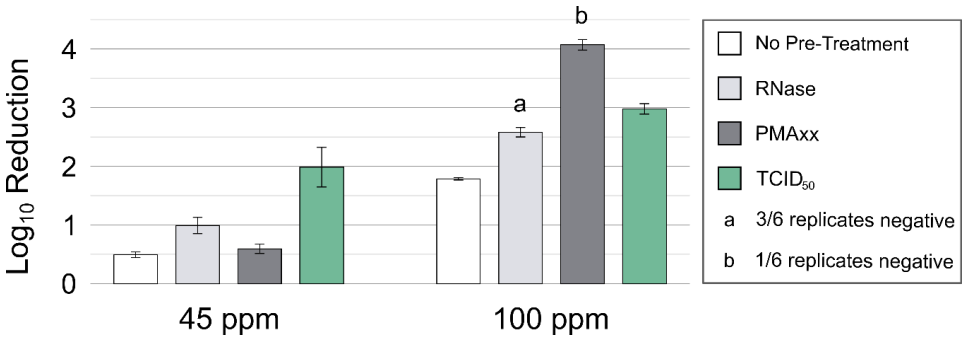

Figure 19: Log_{10} reduction in TuV after chlorine treatment with 45 and 100 ppm as detected by two-step RT-qPCR = Lo RT. Log_{10} reductions are depicted for PCR analyses without pre-treatment (☐), with RNase (▢), or PMAxx (▩) and for $TCID_{50}$ (▩); error bars represent standard errors. (a) 3/6 replicates negative in qPCR; (b) 5/6 replicates negative in qPCR.

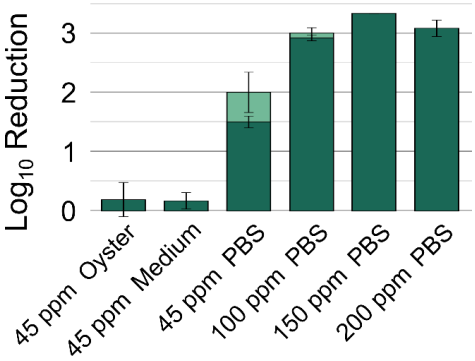

Figure 20: Log_{10} reduction in TuV titre in oyster, cell culture medium, and 1:10 diluted in PBS after exposure to chlorine. (▩) Reductions during method development in Paper I; (▩) Reductions in the context of the chlorine pilot in Paper IV; error bars represent standard errors.

4.4 MECHANISMS OF VIRUS INACTIVATION

We have observed that the strategies applied in this work to mitigate NoV in oysters show varying success in the inactivation and removal of the pathogen (Papers II-IV). This may be related to the different mechanisms of inactivation and the effect they have on the virus. The virus capsid can sustain damage to varying degrees. In case of minor damage, only the structure of the capsid proteins may be altered. This may lead to changes in receptor binding capacity or antigenicity. Thus, one could expect to observe a reduction in virus infectivity in cell culture, whereas there would be no effect of PMAxx and no reduction in RNA. If the capsid disruption is more extensive, the structure could be completely compromised. In that case, a reduction in infectious virus would be observed in cell culture, and there should also be an effect of PMAxx. The dye can reach the genome if the capsid is severely damaged and inhibit genome amplification. In addition, the disruption of the capsid would allow environmental RNases to reach the genome and degrade the RNA. Consequently, the amount of genomic material that is detected in RT-qPCR should decrease. Keeping that in mind, we can now have a closer look at the mechanisms of virus inactivation that may have occurred during our experiments (Papers II, III). The mechanism of virus inactivation after thermal exposure has been examined previously (Hirneisen et al., 2010). Here, this inactivation mechanism is described in relation to our observations. In addition, the fate of infectious virus in oysters during depuration is addressed.

Elevated temperatures act mainly on the virus capsid. A change in isoelectric point, conformational changes in secondary and tertiary structure, as well as rupture of the capsid have been reported by Hirneisen et al. (2010) and references therein. Accordingly, we observed a strong effect of the PMAxx pre-treatment if TuV was exposed to increasing temperatures (Paper I). During thermal treatments in Paper II, PMAxx was less advantageous for samples on the disposable barbecue, which reached lower temperatures than those on the gas grill or in the oven. Thus, it is plausible that capsid damage increases with rising temperatures. In addition, this capsid damage leads to a decrease in infectivity in cell culture, which we observed for all heat-treated samples (Papers I, II). At the same time, we observed a reduction in TuV RNA. At the mildest heat treatment in Paper I, and after the disposable barbecue in Paper II, only a small decrease in RNA was observed. More substantial reductions were observed after the gas grill and broiling (Paper II). This indicates

that the higher the temperature, the more the genome is affected as well. It follows that heat induces severe capsid damage and can additionally act on the genome (Figure 21).

In contrast to heat-treated viruses, there was no benefit of the PMAxx pre-treatment for the depuration samples (Paper III). There must have been only mild capsid damage during the experimental period, which did not facilitate PMAxx entry (Figure 21). Still, the capsid must have been affected by some means since we observed a significant decline in TuV infectivity over the course of four weeks. Hypothetically, the virus capsid may have been attacked by enzymes in the oyster DT. These enzymes are present in the oyster digestive system and are also found in haemocytes, in which NoV can be detected (Boucaud et al., 1983; Xue and Renault, 2000; Yonge, 1926). Enzymes mainly found in haemocytes comprise aminopeptidases and various glycosidases, among others (Xue and Renault, 2000). The same enzymes as well as proteases and lipase have been found in the different parts of the digestive system of Pacific oysters (Boucaud et al., 1983). Glycosidases seem to be abundant in the oyster, but it has been demonstrated that gelatine was liquified after two to six days in the presence of extract from the diverticula, which indicates the presence of proteases (Yonge, 1926). Therefore, one could assume that prolonged exposure of TuV and NoV to proteases in the oyster DT during depuration may have led to changes in capsid structure. Deng and Cliver (1995) suggest that bacterial proteases lead to a reduction in infectious HAV. HAV infectivity decreases in the presence of bacterial isolates from manure, but this HAV inactivation is in some cases inhibited if protease inhibitors are added. This indicates bacterial proteases are responsible for HAV inactivation. Similarly, proteases have a damaging effect on NoV GII.4 VLPs but may not influence NoV capsids in the same manner (Chassaing et al., 2020). Chassaing et al. (2020) concluded that the GII.4 capsid was not affected by the treatment with proteolytic enzymes as an RNase pre-treatment did not have any effect. Thus, capsid damage must have been minimal. Likewise, the capsid cannot have been severely affected during our depuration trial as the TuV genome seems to have been well-protected. As discussed above (4.3.1), TuV RNA was continuously detected during the depuration trial, although TuV infectivity clearly decreased in cell culture. The decrease in infectious TuV in cell culture may be a result of reduced host cell binding capacity.

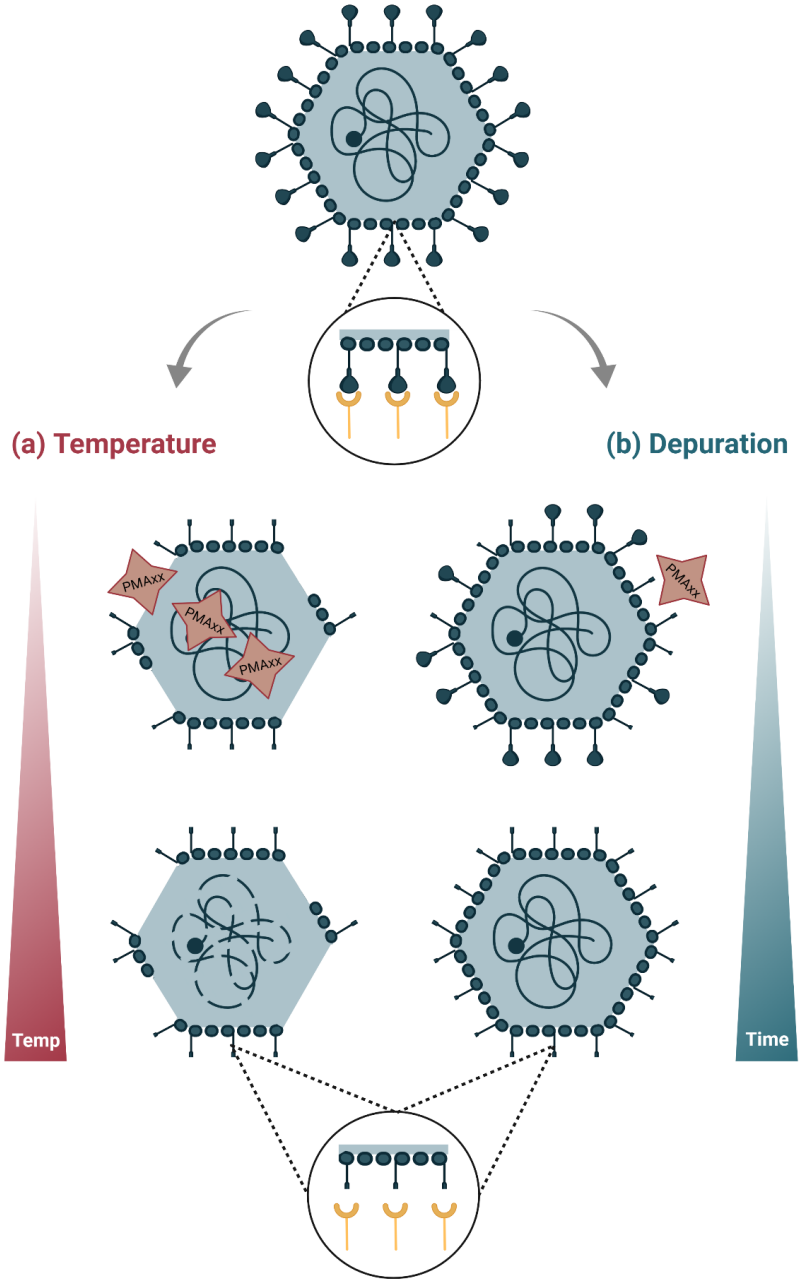

Figure 21: Hypothetical inactivation mechanism of viruses due to elevated temperatures (a) and during oyster depuration (b). In its infectious state, the virus binds to host cell receptors (☽—) via its binding domain (●—). (a) Exposure to heat disrupts the capsid, so that PMAxx (✦) can enter. Further heat exposure affects the genome, which would explain the benefit of long-range PCRs. (b) Conditions during depuration act less severely on the capsid, which does not allow PMAxx entry. Still, binding to host cell receptors is disrupted. Figure created with BioRender (2017).

4.5 IMPLICATIONS OF OBSERVED VIRUS REDUCTIONS FOR FOOD SAFETY

Whether any of the heat treatment or depuration conditions assessed for virus reduction in the present work (Papers II, III) will result in a safe food product, will depend on the initial viral load. Natural contamination levels are in general low. Guix et al. (2019) give an overview of the viral load in oysters and other shellfish by region. Oysters in Europe seldom carry $>10^4$ GC of NoV per gram DT. In several publications that provide data on NoV contamination in oysters, the average NoV concentration lies below 1,000 GC/g (Figure 22). Still, Battistini et al. (2021a), Lowther et al. (2012b) and Suffredini et al. (2014) report average levels of 3,100, 2,243 and 2,000 GC/g, respectively, in oysters that were harvested mainly in class B areas.

Lowther et al. (2012a) determined that NoV-positive oysters were involved in outbreaks when the viral load was ~1,000 GC/g on average (150-8,200 GC/g). The probability of causing infection was also linked to viral load, as 75% of outbreak samples were contaminated with >500 GC/g. In contrast, non-outbreak oyster samples carried 120 GC/g on average. No outbreak was reported when NoV concentrations were <100 GC/g. Considering these numbers, NoV reductions observed during our depuration trial (Paper III) may lead to oysters that are safe for human consumption. After one week, about 0.8 $\log_{10}$ of the initial NoV load was removed, and after another week, reductions exceeded 1.0 $\log_{10}$. Consequently, NoV levels in naturally contaminated oysters would be reduced to ~100 GC/g or less, which was a concentration not involved in NoV outbreaks according to Lowther et al. (2012a). Thus, the conditions in our depuration trial may make most naturally contaminated oysters safe for consumption. The same may not be true for heat-treated oysters that carry NoV (Paper II). After grilling and broiling, NoV levels decreased by <1.0 $\log_{10}$, so that food safety cannot be guaranteed.

Here, we can also include data on TuV infectivity. Depuration of at least 14 days reduced infectious TuV by ≥1.0 $\log_{10}$, and ≥4.2 $\log_{10}$ of the initial load were removed after 28 days (Paper III). Similarly, TuV infectivity decreased by 1.2-3.6 $\log_{10}$ after the cooking procedures in Paper II. We demonstrated 5-8 min on the gas grill or in the oven (broiling) reduced infectious TuV by >3.0 $\log_{10}$. Heat exposure time on the disposable barbecue may be prolonged to be on the safe side, as after 8 min, a 1.2 $\log_{10}$ reduction was observed.

Assuming NoV infectivity decreases in a similar fashion as that of TuV, the risk of NoV infections will be minimal after these treatments. However, at low levels of NoV contamination, the rate of depuration may be slower than what we observed for TuV infectivity reductions in Paper III, in which the initial TuV load was $>10^4$ $TCID_{50}$/mL. Moreover, at elevated levels of infectious NoV in oysters, as encountered after recent contamination or during winter (Battistini et al., 2021a; Lowther et al., 2012b), the reductions observed in the present work may not suffice to ensure a safe product. Battistini et al. (2021a) report the highest NoV level of 22,000 GC/g in February and in EFSA's 2019 baseline survey 1.2% of samples were contaminated with >5,000 GC/g. In these cases, depuration may have to be extended to one month, as we observed a >4.0 log_{10} reduction in infectious TuV after this time. Broiling and the gas grill may still lead to a safe product, but heat exposure times may still be extended for a safety margin. Combining both depuration and heat treatment should suffice to obtain a safe product. For instance, after 14 days of the 17°C depuration, TuV infectivity was reduced by 2.6 log_{10}, and cooking, even on the disposable barbecue, reduced TuV by ≥1.2 log_{10}. Thus, the combined reduction of ≥3.8 log_{10} from the two processes would significantly reduce the risk of a virus infection.

If reductions in TuV and NoV infectivity were comparable, and contamination levels of oysters mirrored the average NoV concentrations in Figure 22, a two-week depuration as well as grilling and broiling of contaminated oysters would result in a safe product. Still, there is some indication that NoV is not as affected by heat as TuV. We observed that reductions in NoV RNA were smaller than those in TuV RNA (Paper II). The NoV capsid may also be less sensitive to heat than the capsid of TuV as the effect of PMAxx was greater for the latter (Paper II, Figure 14). It was already indicated that NoV may be more resilient to heat than surrogate viruses (Escudero-Abarca et al., 2014; Jeon et al., 2020; Knight et al., 2016; Li et al., 2017; Topping et al., 2009). NoV GII.2, the genotype that was worked with in the present project, has also been recently reported to be less affected by heat than TuV and GII.4 (Tan et al., 2021). Thus, it is not easy to extrapolate from surrogate viruses to NoV itself. Although surrogates in the *Caliciviridae* are similar in structure, genome composition and organisation, their tolerance to inactivating conditions may vary. The reductions in NoV infectivity in oysters should be further examined, for instance by utilising the HIE system, to make sure NoV and TuV are comparable in that regard.

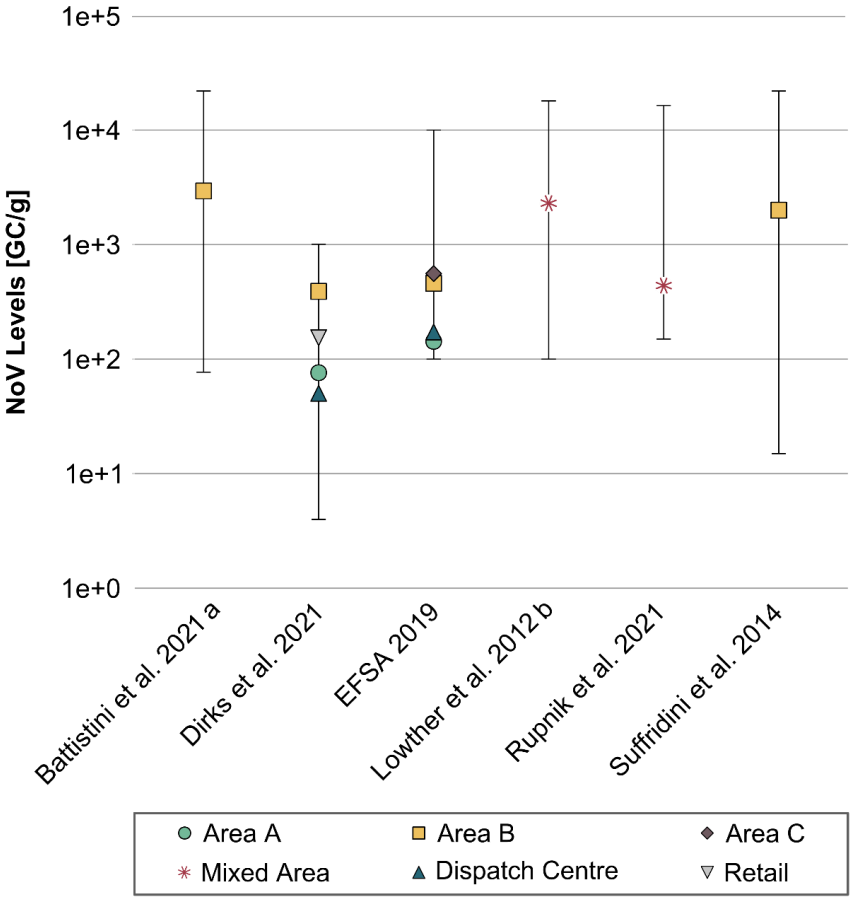

Figure 22: NoV levels in oysters in genome copies (GC) per gram oyster tissue; average NoV levels are depicted, and bars represent the range of the NoV load. Minimum NoV levels for Battistini et al. (2021a) and Lowther et al. (2012b) are given as below the limit of quantification (LOQ) or <100 GC/g. In Lowther et al. (2012b), average NoV levels differed greatly between sampling sites (50-2,243 GC/g) and only the highest value is depicted. The maximum level in EFSA (2019) is given as ≥10,000 GC/g. ✳ Mixed Area = Oysters were harvested from class A, B and/or C areas.

5 CONCLUSION

Shellfish are commonly viewed as culinary delicacies, giving high retail prices. This is especially true for several types of bivalve molluscs. Among those, raw oysters are the pinnacle of fine dining. Still, this high-class product is often associated with food-borne illness, particularly with NoV infections.

This study highlights that appropriate heat treatment during cooking of contaminated oysters can help mitigate the risk of NoV infection. An average viral load as reported by EFSA for naturally contaminated oysters would no longer pose a risk to the consumer after broiling or grilling, which would make even recreationally harvested Pacific oysters a safe food source. However, such thorough heating will lead to a certain loss of organoleptic quality. For the optimal gastronomic experience oysters that can be assured free of virus and served raw or mildly heated would be desirable.

We demonstrated that a rise in water temperature can be an appropriate means to facilitate the removal of viruses during depuration. Infectious TuV was reduced by >2.5 $\log_{10}$ after two weeks at 17°C, while at 12°C reductions were still <2.0 $\log_{10}$ after three weeks. After one week, reductions were not noticeably different. These results imply increasing the water temperature may be relevant for prolonged depuration. Keeping the water temperature >10°C year-round and extending the depuration time to one week or more should minimise the risk of infection after the consumption of raw oysters contaminated in their natural habitat. In contrast, the application of disinfecting agents as hypochlorite does not seem to increase the removal or inactivation of virus in contaminated oysters. In our chlorination pilot, no effect of 45 ppm chlorine on TuV infectivity was observed, despite a 1.5 $\log_{10}$ reduction in infectious TuV in PBS after exposure to the same chlorine concentration. Applying chlorine during depuration may be challenging due to the rapid decline in chlorine concentration in the presence of organic matter, a decreased efficacy at low temperatures, and the formation of by-products. Elevated chlorine concentrations may also lead to an undesirable taste. Chlorine is also likely to lose a considerable amount of activity before it enters the oyster and reaches its target in the digestive tissue.

To properly evaluate the efficacy of the described depuration and shellfish processing strategies in reducing the load of infectious NoV, it is crucial to determine the number of

infectious viruses in oyster tissue. As the cultivation of NoV in cell lines has not been successful and the HIE system is challenging to establish, RT-qPCR modifications may help determine the loss of infectivity during depuration and post-harvest processing. We have demonstrated that the addition of pre-treatments like PMAxx prior to RNA isolation and RT-qPCR analysis can reduce amplification of thermally inactivated TuV with damaged capsids, as results closely resembled cell culture reductions. If the genome was affected as after UV exposure, long-range PCR reductions did not differ from those detected in cell culture. After further optimisation to make this method more dependable, Lo PCR may be applied if genome damage of viruses is to be expected. Overall, applying pre-treatments and long-range PCRs can offer an opportunity to reduce amplification of RNA from inactivated viruses. However, the advantage of these approaches seems to be dependent on inactivation, and each method would need to be optimised for the individual type of inactivation.

6 FUTURE PERSPECTIVES

The present work has illustrated that a modification in molecular analyses can be an advantage when it is desired to only detect infectious virus. If these methods could be streamlined into one method that performs reasonably well for a variety of inactivation conditions, screening for infectious NoV would be simplified. A molecular method is desirable for this, as the application of HIEs, or possibly cell lines in the future, would be too time-consuming for routine analysis. If such a reliable method were to be established, results from NoV inactivation studies would be put into perspective, and bivalve safety as well as the effect of various depuration techniques could be better assessed. In case such a method contained a long-range PCR step, a shorter target sequence is worthwhile to explore and might lead to more stable results. A sequence of ~1 kb may be easier to reverse transcribe or amplify compared to the 1.5 and 2.1 kb in the present study and may already detect a reduction in infectivity due to genome damage (McLellan et al., 2016). With a functional long-range PCR, the impact of UV light on the virus genome during different times of the year could be evaluated. It is possible that during summer, NoV RNA sustains more damage. That would reduce the risk associated with oyster contamination at that time of year and could be interesting to investigate with a long-range method.

The development of a molecular assay that reliably detects only infectious NoV would also shed further light on NoV inactivation within oyster tissue during cooking. In this respect, the number of cooking procedures evaluated for their inactivation capability should be expanded and, on the same note, different NoV genotypes should be included in future work. The incorporation of several NoVs would further elucidate the distinct behaviours of each strain and would form a more comprehensive picture of the genus. Ideally, future studies should also focus on working with naturally contaminated oysters. Thus, realistic contamination levels and the most relevant NoV genotypes would be included.

In terms of post-harvest processing, modifications in the depuration process should be further investigated. Elevated temperatures seem like a valid approach, although the costs of heating should be compared to costs of prolonged depuration. In this regard future work should mainly focus on the advantage of water temperatures above 10°C (Kingsley et al., 2018; Rupnik et al., 2021). A benefit of temperatures above 10°C would be of special

relevance in Scandinavia where water temperatures during winter months can be lower than 5°C. Thus, any studies done in this temperature range would be of great value to the Norwegian seafood industry, who could adjust depuration procedures accordingly. In addition, future depuration studies should be conducted during winter not only due to the higher prevalence of NoV at that time of year, but also because of the seasonality of receptor expression in oyster tissue (Maalouf et al., 2010).

In the context of depuration, the hypothetical loss of host cell binding during the process could be further investigated as well. For this, a cell-binding assay could be developed. In addition, digestive enzymes could be extracted from the oyster to examine if they affect the virus in terms of cell binding. Such analyses would shed light on the fate of NoV in oysters during depuration.

Although oyster chlorination was unsuccessful in our trial, the potential of applying disinfecting compounds during depuration could still be further researched. These compounds should be less harmful than chlorine, which forms concerning by-products in the presence of organic matter. Natural agents have shown promise in reducing NoV surrogates. Among them are grape seed extract, essential oils, like lemongrass oil, polyphenol compounds in red wine, and green tea extract (Falco et al., 2019; Joshi et al., 2015; Kim et al., 2017; Oh et al., 2015b). Chitosan could be considered as well (Davis et al., 2015; Fang et al., 2015). In this context, the transport of the active agent into the oyster digestive tract should also be focused on. To achieve this, alginate microcapsules which carry antimicrobial agents could be considered (Darmody et al., 2015). Recently, Gorji and Li (2022) reported a decrease in TuV when chitosan-coated alginate beads carried a photosensitiser into the DT of Pacific oysters. Regardless of modifications, the ideal approach should also be as cost-effective as possible so that it could be used in commercial depuration facilities without being too much of an economic burden on the industry.

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APPENDIX

Primers & Probes

Appendix - Table 1: Primer and probes. TV_LR primer and probe were designed in primer BLAST and used for TuV long-range RT-PCR and long-range RT. TVIF (Drouaz et al., 2015) was used for TuV RT-qPCR and qPCR. COG2R, QNIF2d and QNIFSP (Kageyama et al., 2003; Loisy et al., 2005) were used for NoV GII. For RT-ddPCR, QNIFSP and TVIF probes were modified with a ZEN/Iowa Black FQ quencher.

Oligonucleotide	Sequence (5'-3')
TV_LR_f	AGATTTGTCCGCCCAAGAGG
TV_LR_r	GTGGTACGGGAGGAAGTGTG
TVIF_f	CTGGGATACCCACAACATC
TVIF_r	GCCAGTTAACAGCTTCAGC
TVIF_p	FAM-TGTGTGTGCCACTGGATAGCTAGCACC-BHQ
COG2R	ATGTTCAGRTGGATGAGRTTCTCWGA
QNIF2d	TCGACGCCATCTTCATTCACA
QNIFSP	JOE-AGCACGTGGGAGGGCGATCG-BHQ

Kits

Appendix - Table 2: Kits and vendors.

Kit	Supplier
Maxima Probe qPCR Master Mix with Separate ROX Vial	Thermo Fisher Scientific Waltham, MA, USA
One-Step RT-ddPCR Advanced Kit for Probes	Bio-Rad Hercules, CA, USA
OneStep RT-PCR Kit	Qiagen Hilden, Germany
QIAamp Viral RNA Mini Kit	Qiagen Hilden, Germany
QIAquick PCR Purification Kit	Qiagen Hilden, Germany
RNA UltraSense One-Step Quantitative RT-PCR System	Thermo Fisher Scientific/Applied Biosystems Waltham, MA, USA
SuperScript IV Reverse Transcriptase	Thermo Fisher Scientific/Invitrogen Waltham, MA, USA
TaqMan Fast Virus 1-Step Master Mix	Thermo Fisher Scientific/Applied Biosystems Waltham, MA, USA

Chemicals

Appendix - Table 3: Chemicals and vendors.

Product	Supplier
Antibiotic-antimycotic (100x)	Thermo Fisher Scientific/Gibco Waltham, MA, USA
dNTP Mix, PCR grade (2.5 mM)	Thermo Fisher Scientific/Invitrogen Waltham, MA, USA
Foetal bovine serum, Qualified, Brazil	Thermo Fisher Scientific/Gibco Waltham, MA, USA
Medium 199, GlutaMAX supplement	Thermo Fisher Scientific/Gibco Waltham, MA, USA
NucliSens Lysis buffer	bioMérieux Marcy-l'Étoile, France
NucliSens Magnetic extraction reagents	bioMérieux Marcy-l'Étoile, France
PMAxx Dye (20 mM)	Biotium Fremont, CA, USA
Proteinase K, Recombinant, PCR grade (~20 mg/mL)	Thermo Fisher Scientific Waltham, MA, USA
Recovery cell culture freezing medium, DMSO (10%)	Thermo Fisher Scientific/Gibco Waltham, MA, USA
RNase A, DNase and protease-free (10 mg/mL)	Thermo Fisher Scientific Waltham, MA, USA
RNase inhibitor (20 U/μL)	Thermo Scientific/Applied Biosystems Waltham, MA, USA
Sodium hypochlorite/sodium hydroxide Stock solution (15%)	Ambio AS Stavanger, Norway
Sodium thiosulfate, Purum p.a., anhydrous, ≥98.0% (RT)	Merck/Sigma Aldrich Darmstadt, Germany
Trypsin-EDTA (0.05%), Phenol red	Thermo Fisher Scientific/Gibco Waltham, MA, USA

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