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Exploring the association between viruses, microplastics and biofilms, and the impact of exposure to an *in vitro* digestion model

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Abbreviations

BBL-tubes	Bashing Bead Lysis Tubes
BCoV	Bovine coronavirus
CPD	Critical point drying
CPE	Cytopathic effect
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
EPS	Extracellular polymer substance
Exp	Experiment
GI	Gastrointestinal
H/h	Hour(s)
HAV	Hepatitis A virus
HuNoV	Human norovirus
IEP	Isoelectric point
LOD	Limit of detection
LPS	Lipopolysaccharide
MP	Microplastic
NC	Negative control
NMBU	Norwegian University of Life Sciences
PBS	Phosphate-buffered saline
PC	Positive control
PE	Polyethylene
PP	Polypropylene
RGE	Rabbit gastric extract
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Room temperature
RT-ddPCR	Reverse transcription digital droplet polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
S/s	Second(s)
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SSF	Simulated salivary fluid
$TCID_{50}$50%	Tissue-culture infectious dose
TEM	Transmission electron microscopy
TV	Tulane virus
VLP	Virus-like particle
xG	Times gravity

Sammendrag

På grunn av den økende spredningen av plast og mikroplast i verdens økosystemer, øker den menneskelige eksponeringen for og konsumet av mikroplast. I miljøet blir mikroplast kolonisert av forskjellige mikroorganismer, både humant patogene og apatogene. Koloniseringen av mikroorganismer på mikroplast, har fått tilnavnet «plastisfærer». Det finnes mye kunnskap og litteratur angående human matforgiftning med planktoniske bakterier, virus osv. Imidlertid mangler det kunnskap om konsekvensene av inntak av plastisfærer og om dette kan medføre en risiko for vann- og matbårne infeksjoner, og dermed en mulig smittevei for mennesker. Derfor er det viktig å undersøke helsekonsekvensene av menneskelig inntak av plastisfærer. Mitt prosjekt har fokusert på eksperimenter der vi har undersøkt hvordan Tulane virus og bovint coronavirus fester seg på plast både med og uten biofilm, og om virusene er i stand til å bli en del av plastisfærer der biofilmen er laget av mikroorganismer i elvevann. I tillegg har vi undersøkt virusstabiliteten i plastisfærene etter de ble sendt gjennom en *in vitro* fordøyelsesmodell, ved å bruke RT-qPCR og RT-ddPCR. Resultatene viser at begge virus fester seg til plast og kan bli en del av plastisfæren, og at de kan oppdages på forskjellige tidspunkter underveis i fordøyelsesmodellen samt i biofilmsedimenter etter endt fordøyelse. Med denne oppgaven ønsker vi også bidra med verdifull informasjon for de som vil begi seg ut på lignende forsøk i framtiden, da den belyser at arbeidet med disse metodene har vært lærerikt, men ikke alltid like lett.

Summary

Human ingestion of and exposure to microplastics (MPs) are increasing due to the vast spread of MPs in various ecosystems and plastic particles existing all around us. MPs are easily colonized by both human pathogenic and apathogenic microorganisms in the environment, creating small microbial communities called plastispheres. The body of knowledge regarding food poisoning with ingestion of different planktonic bacteria, viruses etc. is large. However, there is lacking knowledge on what happens with the microorganisms in plastispheres when humans ingest them, and whether this provides a pathway for food- and waterborne infection and thereby disease. Therefore, the importance to investigate the consequences ingestion of plastispheres might have for human health is significant. My project focused on how Tulane virus (TV) and bovine coronavirus (BCoV) would attach to plastic pieces both with and without biofilm, and if they were incorporated in biofilms created by environmental microorganisms found in river water on plastic pieces. In addition, we have investigated the viral stability in the plastispheres when being sent through an *in vitro* digestion model, by executing RT-qPCR and RT-ddPCR. Results show that both viruses attach to plastic, become a part of the plastisphere, and have been found throughout the digestion model and in biofilm sediments after finished digestion. Finally, because of the many challenges faced underway during this work, this thesis provides valuable information for others that might want to carry out experiments with viruses, plastispheres and the *in vitro* digestion model.

1 Introduction

1.1 Plastic pollution and human exposure to plastics

In recent years, an invisible threat has been steadily infiltrating our environment and our food chain. MPs, minute particles less than five millimeters in diameter, often go unnoticed by the naked eye (Frias & Nash, 2019). Yet, they are dispersed all over nature, and excessive amounts are found in the world's oceans and shores (Cole et al., 2011; GESAMP, 2015; Geyer et al., 2017). Worldwide, an estimated total of 19 to 23 million metric tons of macro- and microplastic combined enter the aquatic environment (Borrelle et al., 2020). When plastics become exposed to factors such as UV radiation, mechanical stress, and chemical and biological degradation, they fragment into MPs. These are called secondary MPs. Primary MPs are pieces manufactured as MPs and found in, among other things, personal hygiene products and synthetic fabrics (Wu et al., 2022). The most produced plastic polymers are high- and low-density polyethylene (PE), polypropylene (PP) and polyvinyl chloride (OECD, 2022). This is consistent with the findings that PE and PP are the most abundant MPs in the marine environment (de Haan et al., 2019).

Because of the rising number of MPs in the ecosystems and the migrating MP debris from food packaging, human exposure to and consumption of MPs are set to increase (Cox et al., 2019; Jadhav et al., 2021). MPs can be ingested by aquatic organisms at lower trophic levels, bioaccumulate, enter the food chain and thereby pose a potential risk to human health (Li et al., 2018; Su et al., 2019). It is estimated that human adults annually consume about 50000 and inhale up to 62000 microplastic particles (Cox et al., 2020). In 2021, the first MPs were discovered in human placentas, signaling that MPs might be ingested and taken up into the human bloodstream and spread throughout the body. However, the mechanisms for exactly how the MPs end up in the human bloodstream are still unclear. The most likely place for the uptake is either the gastrointestinal (GI) or respiratory tract. Effects of MPs in the bloodstream on human health are still yet to be decided (Ragusa et al., 2021). In rats, it has already been shown that inhaled MPs translocate across the pulmonary circulation, and deposit in the placenta and fetal tissues (Fournier et al., 2020). There is proof for the translocation of MPs across the GI tract and into the circulatory system in mussels. MPs were detected in these mussels' circulatory systems for 48 days but might have been detected for even longer if the experiment had been continued (Browne et al., 2008; Revel et al., 2018).

So, it is established that humans ingest and inhale MPs, and we know that somehow MPs end up in our bloodstream in addition to being excreted through feces (Schwabl et al., 2019;

Zhang et al., 2021). What are the potential consequences of having MPs circulating in our bodies? This is a field of research where there is still a lot of uncharted territory. Most research on the ingestion of MPs has been performed on marine organisms, so the body of knowledge regarding its effects on humans is limited (Blackburn & Green, 2022). Some MPs have been shown to have cytotoxic effects through increasing oxidative stress on human epithelial and cerebral cells *in vitro*, and PP in direct contact with human-derived cells caused releases of cytokines and histamines, indicating a pro-inflammatory effect (Hwang et al., 2019; Schirinzi et al., 2017). The ingestion of MPs themselves could have several adverse health effects, but there is still a lack of consensus regarding whether it is the plastic itself or the additives in the plastics that are the most toxic (Blackburn & Green, 2022). Additives are different chemical compounds that are added to plastic polymers during their production to alter and adapt their physical and chemical properties (Rochman, 2015). However, research has shown that without the additives, "clean" plastic does not cause toxic effects (Kühn et al., 2020; Sørensen et al., 2023).

1.2 Viral food- and waterborne infections

Globally, viruses in drinking water and food are major causes of water-and food-borne infections with corresponding development of illness in humans. Many viruses spread rapidly between hosts and are especially contagious in closed environments like nursing homes, schools, hospitals, and cruise ships. Most prominent is the human norovirus (HuNoV), which is responsible for approximately one-fifth of all acute gastroenteritis outbreaks in the world (Ahmed et al., 2014; Bartsch et al., 2016; Rajagopalan & Yoshikawa, 2016). In addition, HuNoV is the world's leading cause of food-borne illness, as well as the leading cause of death among food-borne diarrheal diseases (Kirk et al., 2015). Risk products when it comes to food-borne infection are foodstuffs like fruit and vegetables, which are often not heat treated before consumption. Through unclean irrigation water, these may become contaminated with human pathogens such as HuNoV, which provides a pathway for food-borne infection and illness (Steele & Odumeru, 2004). Oysters and other bivalves are also risk products if they are not heat treated before consumption, because they continuously filter their surrounding water, leading to an accumulation of pathogens in these organisms (Westrell et al., 2010). HuNoV is also one of the major pathogens that both spreads and infects people through the consumption of polluted drinking water (Gall et al., 2015; Maunula et al., 2005).

Another important food- and waterborne virus which infects humans, is a virus in the enterovirus genus, namely the Hepatitis A virus (HAV). The virus can enter the bloodstream

via the GI tract and infect the liver causing liver disease (hepatitis) (Gall et al., 2015; Tallan & Feng, 2020). Worldwide, approximately 14 million cases of food-borne illness are caused by HAV, yearly, leading to nearly 28.000 deaths (Kirk et al., 2015).

Other notable food- and waterborne viruses include additional enteroviruses and rotaviruses (Koopmans & Duizer, 2004; Steele & Odumeru, 2004). In addition to hepatitis caused by HAV, viruses from the enterovirus genus can cause a wide variety of diseases, from mild, cold-like symptoms, to meningitis, myocarditis and encephalitis (Abzug, 2014; Bruu, 2002). In contrast to enteroviruses, rotaviruses have a more uniform disease manifestation mainly causing gastroenteritis. Despite the development of vaccines, different rotaviruses remain a leading cause of severe diarrhea in children under five years old, causing over 200.000 deaths in this demographic and over half a million deaths globally across demographics (Crawford et al., 2017).

1.3 Biofilm formation and the plastisphere

For over 50 years, it has been known that microorganisms form small communities that protect them from environmental stressors such as antibiotics, biocides and dehydration (Costerton et al., 1978; Donlan, 2002; Garrett et al., 2008; Percival et al., 2011). These communities are commonly known as biofilms. Microorganisms typically found in biofilms are different species of bacteria, algae, fungi, archaea, and protozoa, with bacteria as the main drivers of biofilm formation (Liu et al., 2023). Microorganisms in a biofilm produce an extracellular polymer matrix built up of polysaccharides, proteins, and extracellular nucleic acids. However, the ability to produce these substances varies between different microorganisms (Jefferson, 2004; Kumar et al., 2017). Biofilm can be established on any surface, both biotic and abiotic, whereas plastic is a common one (Bhagwat et al., 2021; Wang et al., 2021). The process of biofilm formation is multifaceted and dynamic, influenced by factors such as cell characteristics, signaling molecules, cell metabolism, and genetic control, and involves five key stages (Kumar et al., 2017; Liu et al., 2023):

1. Initial, reversible attachment to a surface by planktonic bacteria, called primary colonizers.
2. Transition to irreversible adhesion, with the production of extracellular polymeric substances (EPS) by the adhered bacteria. Communication between bacteria through quorum sensing may start.
3. Multiplication and formation of microcolonies lead to an early development of biofilm architecture. The EPS matrix increases in complexity.

4. Further growth and development of more complex three-dimensional structures. Here, different species of bacteria (secondary colonizers) become part of the biofilm.
5. Cell diffusion and dispersion. Pieces of the biofilm are released, and these bacteria return to their planktonic state and might colonize new surfaces. This could happen due to changes in nutrient availability, waste buildup, or other signals.

Biofilms on MPs are formed by different microorganisms in the environment and have gotten the term "plastispheres" (Zettler et al., 2013). The plastisphere protects and facilitates the survival of microorganisms like bacteria, fungi, and algae. Potential pathogenic bacterial species such as *Escherichia coli* and different species from the *Vibrio* genus are also found in plastispheres (Witsø et al., 2023; Zettler et al., 2013). However, it is important to note that not all species of the *Vibrio* genus or strains of *E. coli* are pathogenic (Hossain et al., 2024; Stromberg et al., 2018). In addition, plastispheres seem to be hubs for antimicrobial resistance genes (Yang et al., 2019). Consequently, when plastispheres sneak into our food-chain, for example via shellfish, it may have significant implications for human health (Bowley et al., 2021).

When biofilms are established on foodstuffs and are ingested, there is reason to believe that it may lead to foodborne infections. Bacteria that commonly cause food-poisoning, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *E. coli*, *Pseudomonas* sp., and *Salmonella*, all possess a strong ability to form biofilms. However, little research is done on the effects of the ingestion of biofilms, as almost all pathogenicity research regarding food-borne bacterial infections is done on planktonic bacteria under controlled conditions in the lab (Bai et al., 2021). There is some knowledge regarding the formation of biofilms in different microhabitats of the human GI tract and how this enhances inflammation and plays a part in the development of GI diseases (von Rosenvinge et al., 2013).

1.4 Viruses in biofilms

Viruses have often been, and to some extent continue to be, overlooked as microorganisms that may persist in biofilms. During the late 1990s, increasing attention became directed toward viruses in biofilms. Quignon et al. showed that the persistence of a non-enveloped virus increased when embedded in a biofilm in a pilot drinking water system (Quignon et al., 1997). In 2009, increased persistence was also proved for HuNoV, enterovirus and F-specific phages in wastewater biofilms when compared to only wastewater (Skraber et al., 2009). Since then, not much research has been done in this field.

The attachment, survival and potential infectiousness of viruses present in biofilms and plastispheres have not been properly addressed. How well viruses attach to abiotic plastic depends on several different factors. The isoelectric point (IEP) of the virus, hydrophobic forces of the close environment, and surface roughness are important for virus adhesion (Dika et al., 2015; Dika et al., 2013). In their review, Moresco et al. stated that there is lacking knowledge regarding the risk of food-borne infection via pathogenic viruses attached to MPs and embedded in plastispheres (Moresco et al., 2021). Moresco et al. capitalized on this knowledge gap. In their article published in 2022, they stated that they were the first ones to quantify the potential for virus association in a plastisphere (Moresco et al., 2022), By using surface freshwater from a lake to grow biofilm on MPs (PE) and spiking the water with both a non-enveloped (naked) virus (rotavirus) and an enveloped virus (bacteriophage Phi6), both the enveloped and naked viruses were recovered from the plastisphere. When measuring viral stability and infectivity (plaque assay), the results showed that the infectivity of both viruses from biofilm was higher than for the same viruses in a water phase. Moreover, through the plaque assays, they showed that the naked virus particles kept their infectivity to a larger degree than enveloped viruses, backing the already well-acknowledged claim that enveloped viruses lose their infectivity more rapidly than naked viruses (Casanova & Weaver, 2015; Moresco et al., 2022).

1.5 Viruses used in my study

TV is a small, naked, positive-sense, single-stranded RNA (+ssRNA) virus belonging to the *Caliciviridae* family and *Recovirus* genus, measuring about 36 nm in diameter. This was the first species identified within its genus and the virus was isolated from the feces of captive rhesus macaques. The genome is about 7.5 to 8.5 kb, organized in three open reading frames (ORFs). The ORFs 1-3 respectively code for the nonstructural polyprotein, the capsid protein, and a potentially minor structural protein. There is no known pathogenicity to humans. Because of their similar properties and that TV is easier to cultivate *in vitro,* TV has been utilized in research as a model for HuNoV (Ettayebi et al., 2016; Farkas et al., 2008; Stoppel et al., 2023; Tian et al., 2013). Like HuNoV, TV recognizes human histo-blood group antigens (HBGAs) as their cellular receptors, which makes the virus unique compared to other commonly used HuNoV surrogates such as Feline calicivirus and Murine norovirus (Tian et al., 2013). As previously mentioned, HuNoV is a major cause of viral gastroenteritis among humans on a global scale, particularly among infants and children. We therefore see it as highly relevant to investigate how TV acts in a plastisphere environment, and if its

embedment in the plastisphere could represent a pathway for food-borne infection with HuNoV (Lopman et al., 2016; Payne et al., 2013).

BCoV is an enveloped, single-stranded, positive-sense RNA virus. It ranges from 65 to 210 nm in diameter, and the RNA genome is about 27 to 32 kb, organized into 10 ORFs. The envelope consists of a lipid bilayer and three major surface glycoproteins. These are called M (integral membrane), S (spike) and HE (haemagglutinin-esterase). Functions of these proteins are related to binding to host cells, membrane fusion and spreading of virus from cell to cell. In addition, there is a major non-glycosylated protein called the N (nucleocapsid) protein. This protein is frequently the focus of detection methods due to its high level of conservation across different strains. The virus belongs to the *Coronaviridae* family and is mostly known for causing both respiratory and gastrointestinal diseases in cattle, while it is not pathogenic to humans (Clark, 1993; Saif, 2010; Suzuki et al., 2020). As SARS-COV-2, it belongs to the *Betacoronavirus* genus. Since they belong to the same genus, easily disseminate in a population, have dual tissue tropism for both the GI and respiratory tract and therefore may show similarities regarding clinical signs in their infected hosts, BCoV has been used in research as a model virus for the SARS-COV-2 virus (Shi et al., 2020; Villapol, 2020; Wensman & Stokstad, 2020). Because of this, we also settled on using BCoV as a model virus to investigate embedment in plastispheres.

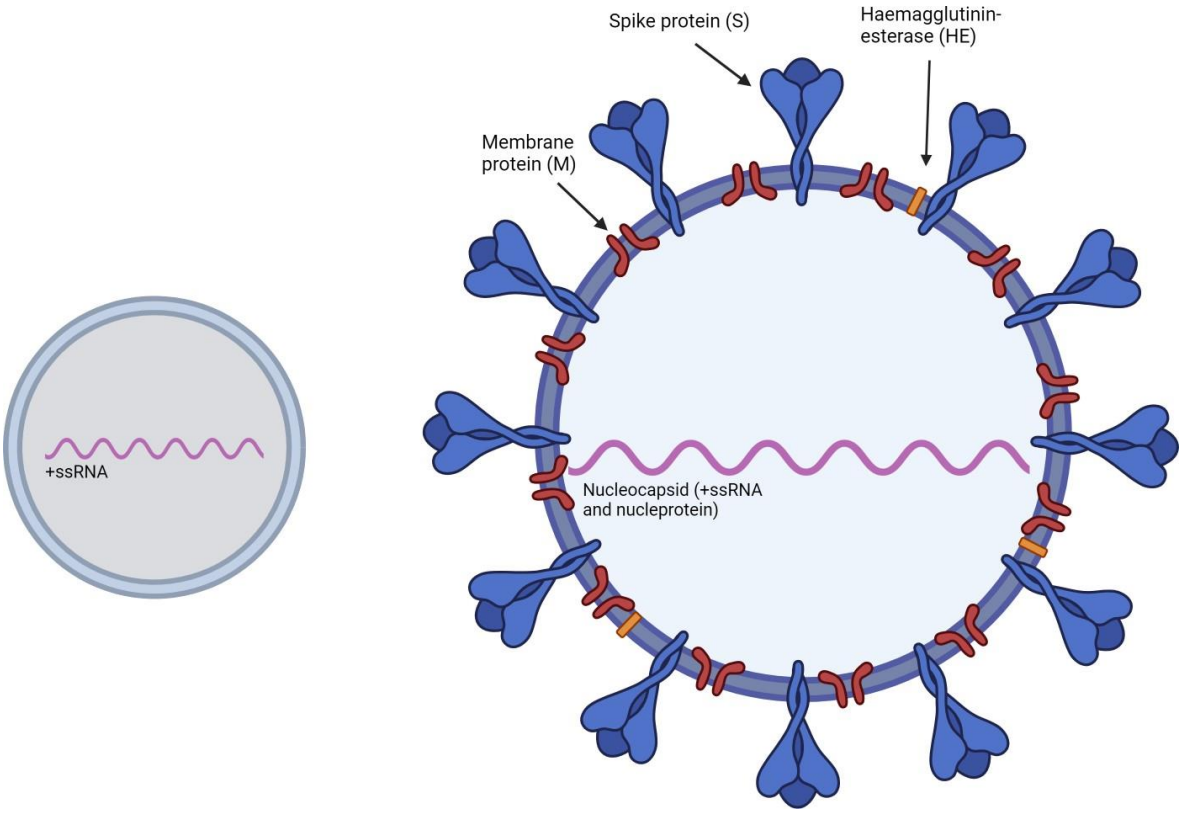


Figure 1: Schematic drawing of both viruses. Tulane virus (TV) on the left, and bovine coronavirus (BCoV) on the right. Created with BioRender.com.

1.6 Rationale

The rationale for performing this study is multifactorial. When plastics and MPs disperse all over nature, different microorganisms will create biofilms on these particles, which are called plastispheres. Viruses pathogenic to humans, such as HuNoVs, enteroviruses and rotaviruses are major causes of food- and waterborne infections, diseases, and deaths. These viruses may become embedded in plastispheres, and there is an indication that viruses may enhance their durability and stability when they are part of a biofilm. Because of the ever-increasing amount of environmental plastic pollution, human exposure to and ingestion of MPs steadily increases. What happens to the plastisphere after ingestion and during digestion, has not been investigated. With a focus on model viruses for potentially food-borne viruses, and by using an *in vitro* digestion model, we seek to answer several questions with this study.

2 Aims of study and hypothesis

Main objective

The main objective of this study is to examine if the two different viruses (TV and BCoV) can become embedded in biofilms made with both specific strains of bacteria and environmental microorganisms, and investigate how such embedment will affect their stability through an *in vitro* digestion model.

Sub-objectives

The main objective is divided into the following sub-objectives:

1. To investigate if TV and BCoV attach to sterile plastic.
2. To investigate if TV and BCoV can become embedded in plastic-associated biofilms made by specific environmental bacteria.
3. To investigate if TV and BCoV become embedded in an environmental biofilm on plastics.
4. To investigate the stability of TV and BCoV in an environmental biofilm, and in the surrounding water.
5. To explore if the *in vitro* digestion model is a suitable method for studying how embedment of viruses in environmental biofilms affects their stability through this model.

Based on these aims, we formulate the following hypothesis: *Both TV and BCoV can attach to plastics, become embedded in plastic-associated biofilms, and are protected by these biofilms during the in vitro digestion model.*

3 Materials and methods

3.1 Virus, bacteria, and plastics

TV, BCoV, and their respective titers ($TCID_{50}$/ml) were provided by the Department of Virology (NMBU). Initial titers were approximately $1.0 * 10^7$ TCID50/ml for TV and $1.12 * 10^6$ TCID50/ml for BCoV. Environmental strains of *Listeria monocytogenes, Pseudomonas putida,* and *Aeromonas hydrophila* were provided by the Department of Food Safety (NMBU). The strains had been isolated from plastic pieces submerged in Lierelva during the PLASTPATH project (Witsø et al., 2023). PP stuffing beads (⌀ 3-5 mm, Fairfield Poly-Fil®, USA) were used for virus adhesion experiments. PP coupons (⌀ 12.7 mm, BioSurface Technologies Corporation, USA) were used for Biofilm reactor experiments.

3.2 Virus adhesion to microplastic pieces

Before working with viruses and biofilms on plastics, we needed to know if TV and BCoV would attach to regular plastic (PP). The first experiments in the project were to investigate how much plastic was needed for the viruses to attach in significant amounts, how the concentration of virus in a solution affected the amount of virus attaching to plastic, and how incubation time affected the attachment.

Amount of plastic. Virus aliquots were thawed on ice. Room-tempered sterile phosphate-buffered saline (PBS, Sigma-Aldrich, USA) was used to make 60 ml of virus solution with an approximate concentration of $1.0*10^4$ TCID50/ml. Ten ml of the solution was added to six ⌀ 50 mm petri dishes (Heger, Norway). Then, 100 mg and 200 mg of PP pieces were added to three dishes each. All petri dishes were incubated at 70 RPM in a rotating incubator (Cole-Parmer ™ Stuart™, USA) at room temperature (RT).

After 24 hours (h), the plastic beads were removed and rinsed twice in sterile PBS, transferred to 15 ml Falcon tubes with two ml of Lysis buffer (NucliSENS® bioMérieux®, France) and stored at -80°C until RNA-extraction.

Incubation time and virus concentration. The effect of these parameters of virus solution on the attachment of TV and BCoV was investigated in different experiments as previously described, with minor modifications. Briefly, for incubation time, 9 × 10 ml of virus solution with an estimated concentration of $1.0*10^4$ TCID50/ml were incubated with 100 mg of sterile PP pieces and incubated for 3, 24, and 48 h in triplicates. To investigate the effect of virus concentration, 6 × 10 ml of virus solution with an estimated concentration of $1.0*10^3$ TCID50/ml were incubated with 100 mg of PP for 3 h and 24 h in triplicates.

For both experiments, samples were rinsed and transferred to Falcon tubes and RNA extraction followed immediately (NucliSENS® miniMAG®).

Investigation of how incubation time and concentration of virus solution would affect BCoV attachment to the plastic was performed in a single experiment, by initially preparing the solution as described previously (6 x 10 ml of $1.0*10^4$ TCID50/ml) with the addition of preparing 6 x 10 ml of a virus solution with an estimated concentration of $1.0*10^3$ $TCID_{50}$/ml. These solutions were added to six petri dishes each. Then, 100 mg of PP pieces were added to each of the 12 petri dishes and incubated for 3 and 24 h. Removal and rinsing of samples were performed as previously described, and RNA extraction followed immediately.

3.3 Virus Attachment to bacteria-coated microplastics

Next, we wanted to investigate how TV and BCoV interacted with the plastic pieces coated with *L. monocytogenes*, *A. hydrophila* and *P. putida*.

The bacteria were plated on blood agar and incubated (aerobically) at 37°C overnight. Successively, one colony from each plate was transferred to a sterile glass test tube with five ml of sterile LB medium and incubated overnight at 37°C (in identical conditions). Then, 50 µl of *L. monocytogenes* and *A. hydrophila*, and 66 µl of *P. putida* culture were added to three different Erlenmeyer flasks containing 20 ml of sterile minimal medium and twelve sterile plastic pieces each. Minimal medium was chosen to mimic river water and prohibit further extensive growth of bacteria. The flasks were incubated overnight at 37°C in a rotating incubator at 90 RPM. The plastic beads were then rinsed with sterile PBS to remove poorly attached bacteria and transferred to petri dishes as previously described. Consequently, 100 mg of plastic beads coated with the bacteria were added to the petri dishes in triplicate, 10 ml of virus solution (described previously) was added, and the petri dishes were incubated at RT 70 RPM in an orbital incubator (Cole-Parmer ™ Stuart™, USA). After three hours, the PP pieces were collected, rinsed in sterile PBS, and transferred to Bashing Bead Lysis Tubes (hereafter referred to as "BBL-tubes", ZymoBiomics™ Zymo Research, USA) containing 400 µl of DNA/RNA Shield™ (ZymoBiomics™). This experiment was repeated twice for TV and once for BCoV.

3.4 Biofilm reactor

3.4.1 Biofilm Reactor Setup

Based on results from a pilot study, a CDC Biofilm Reactor® (BioSurface Technologies Corporation, USA) was used to build biofilm from river water on PP coupons (Figures 2 and

3). The reactor contained eight rods accommodating three coupons each, and a magnetic stirrer. Prior to each experiment, the reactor, rods, and coupons were autoclaved. The same set of equipment was reused for each experiment. A peristaltic Pump System (Masterflex®, VWR™, USA) was assembled by fitting the pump head onto the pump according to the manufacturer's instructions. Tube size 15 was fitted into the pump head and on each side joined with tube size 16 (both tubes from MasterFlex®). A glass container was used as the influent water tank. The water tank was mechanically cleaned with dish soap and tap water, before being left to dry between experiments.

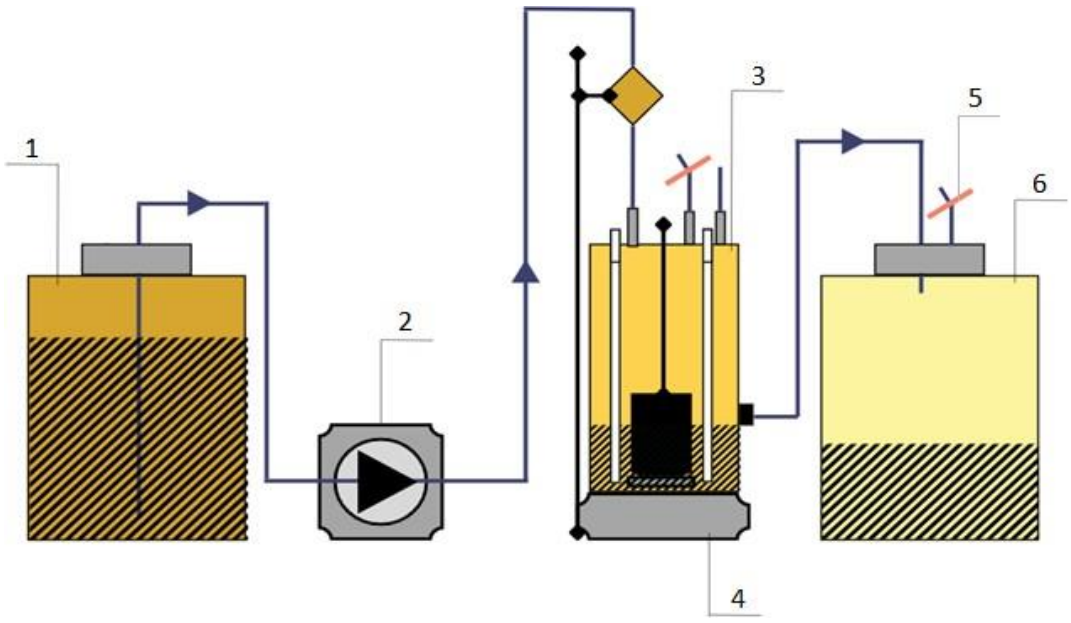


Figure 2 (made by P. Jachimowicz, modified with permission by Hauk): Schematic setup of the biofilm reactor: 1 - glass container with the river water, 2 - pump, 3 – biofilm reactor, 4 – magnetic stirrer, 5 – air filter, 6 - outflow tank.

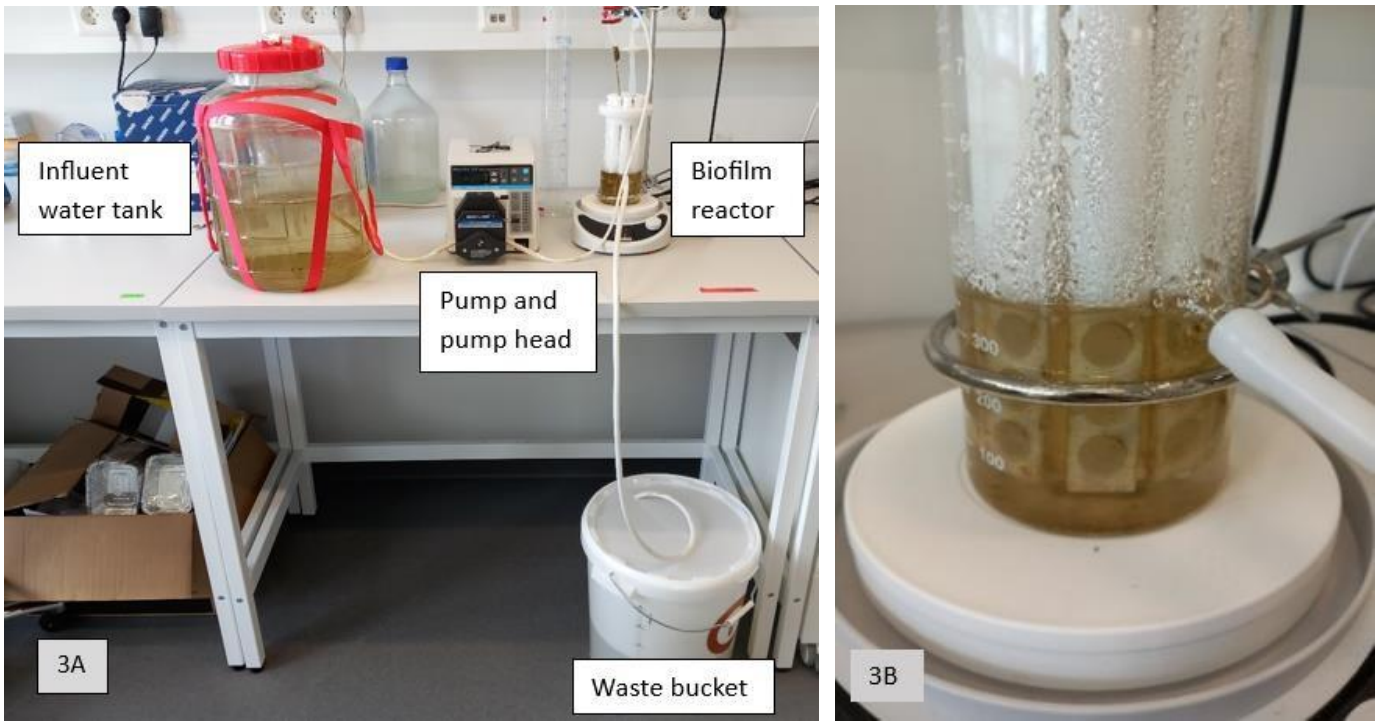


Figure 3A and 3B: 3A; Complete setup of biofilm reactor with pump and influent water tank. 3B; Closeup of biofilm reactor with coupons.

3.4.2 River and collection point

Bølstadbekken located in Ås municipality was used for the collection of water (59°41'21.1"N 10°45'0 7.2"E, red dot, Figure 4). The area surrounding the small river is mostly dominated by agriculture fields and some farms (dairy, horse, and sheep) (Snuggerud, 2013).

Østensjøvannet (black arrow), a small lake uphill from the river, is the starting point for Bølstadbekken, while the larger lake Årungen (red arrow) is the endpoint.

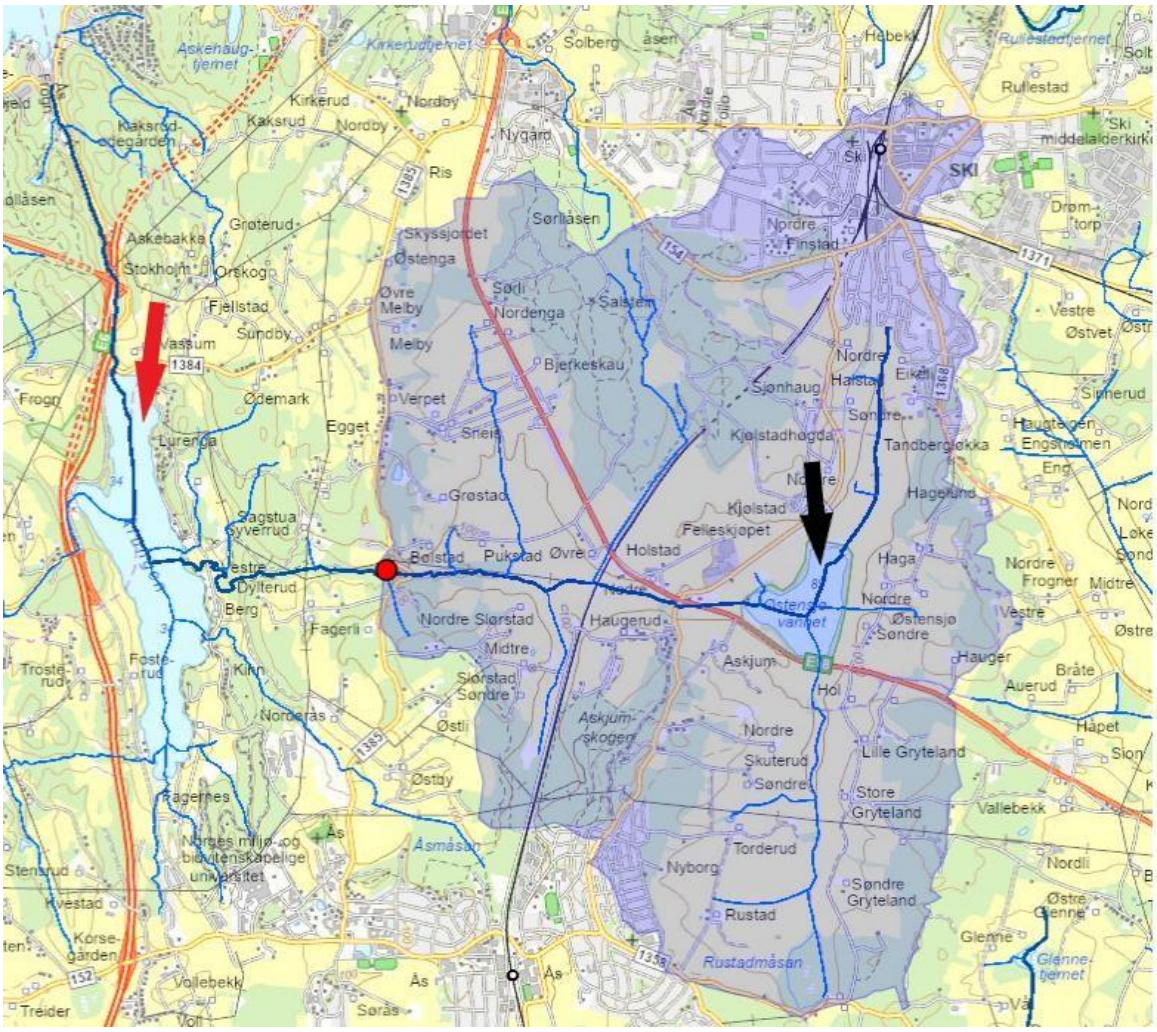


Figure 4: Precipitation area for the collection point (NEVINA)

3.4.3 Collection of river water, spiking with virus and coupon preparation before digestion

Weekly, 20 L of river water was collected and immediately transported to the laboratory within 15-20 min.

All experiments lasted for four weeks (28 days). A five-liter glass container was used for influent water for the first experiment. Due to the small volume of this container, the water had to be changed every two to three days, and collected river water was stored at 4°C in between. The five-liter container was changed to a 20-liter glass container as an influent water tank for the remaining three experiments. Therefore, water changing was carried out weekly during these experiments. Before adding fresh river water, the glass container was rinsed with room-tempered tap water. Each new batch of river water was acclimatized to RT before being transferred to the influent water tank.

The influent water was spiked every second to third day for the first experiment, and weekly for remaining experiments, with TV and BCoV to a final concentration of approximately 200 $TCID_{50}$/ml of each virus. The flow speed of the influent water was set at 1.7 ml/min, and rotation at 100 RPM. After four weeks (28 days) of incubation, the coupons were removed from the bioreactor, rinsed in sterile PBS, and put in 50 ml sterile Falcon tubes. The coupons were used as input material in the *in vitro* digestion model as described in section 2.3.5.

3.4.4 Scanning Electron Microscopy (SEM) of plastic coupons

After four weeks of incubation during the last biofilm experiment, three coupons from the same coupon holder rod were fixed in 2% paraformaldehyde + 1.25% glutaraldehyde in 0.05M PIPES buffer at pH 7. Then, solvent dehydration was followed by critical point drying (CPD) to remove water from the coupons. Finally, the coupons were sputter coated with 15 nm platinum on one side. The negative control (NC) coupon was a previously used coupon, that had been mechanically washed with a brush and dish soap and autoclaved. Fixation and CPD were not performed for the NC. Sputter coating with platinum was performed as with coupon samples.

Pictures were taken with an EVO® 50 SEM (ZEISS).

3.4.5 *In vitro* digestion model

The INFOGEST digestion model is a standardized *in vitro* protocol used to simulate the human digestive system. It consists of an oral phase, a gastric phase, and an intestinal phase (Brodkorb et al., 2019).

Enzyme activity experiments for Rabbit Gastric Extract (RGE15, LipoPolytech, France), Pepsin from porcine gastric mucosa (Sigma-Aldrich, USA), and Pancreatin (Sigma-Aldrich, USA) were executed before digestion experiments, according to enzyme activity assay protocols described in the supplementary information from the INFOGEST Standardized in vitro digestion system (Brodkorb et al., 2019).

Simulated digestive fluids, i.e., salivary fluid (SSF), gastric fluid (SGF) and intestinal fluid (SIF), and purified water (Milli-Q®, Merck, Germany) were preheated in a water bath to 37°C. The settings of a HulaMixer (HulaMixer Sample Mixer, ThermoFisher, USA) were reciprocal movement at 80° for 15 s and vibrating movement at 1° for three s.

As previously explained, coupons from the biofilm reactor were used as input materials for the digestion model. Three coupons were added to each tube and regarded as one sample. We

used a total liquid volume of 1.26 ml in the oral phase, which was enough to immediately cover the coupons. This volume gradually increased as digestion progressed.

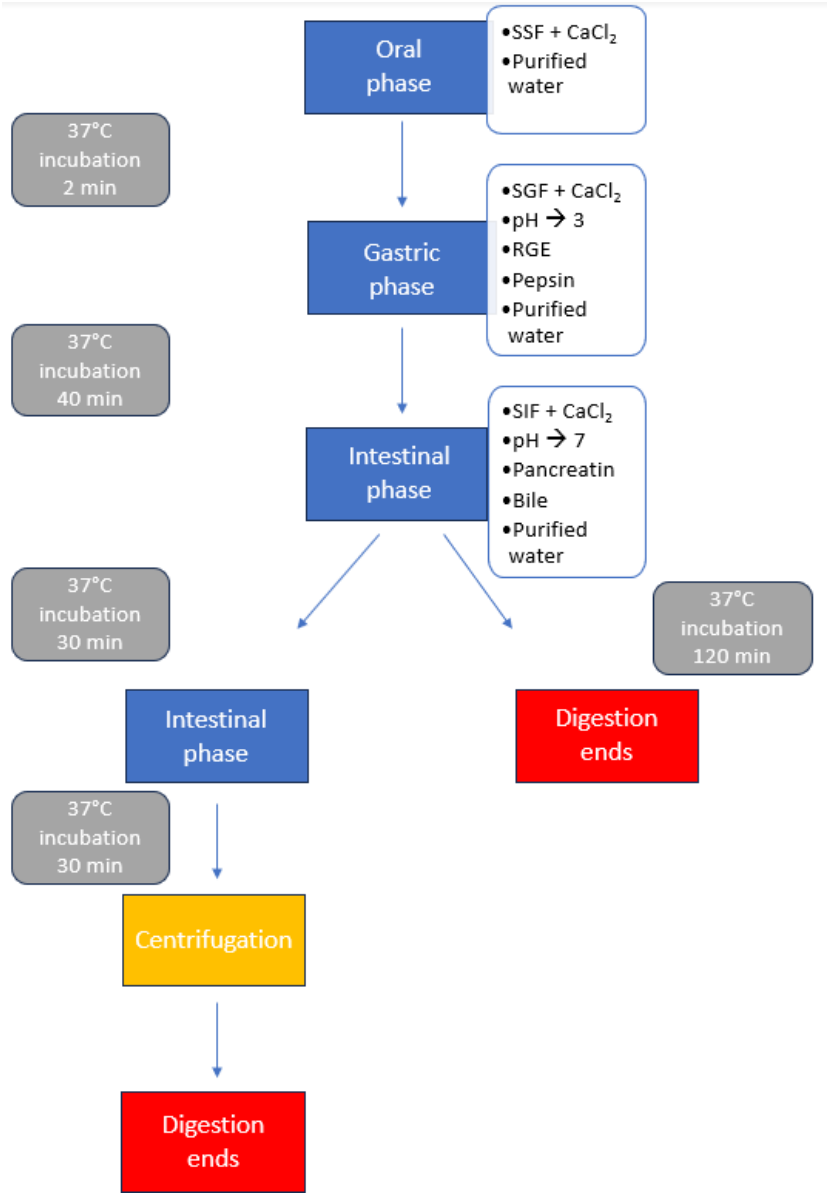


Figure 5: Flowchart of the *in vitro* digestion model.

The different phases in the digestion model are shown in Figure 5 and were executed as follows:

Oral phase

SSF, $CaCl_2$ and purified water were added to the samples. The samples were incubated mounted to the HulaMixer with the abovementioned settings.

Gastric phase

SGF was quickly added to all samples before adding $CaCl_2$. The pH of the samples was adjusted to 3 (± 0.1) using 6M HCl. RGE and Pepsin were added, before validating the pH at

3 and adjusted if needed. Incubation followed, and at the end of the gastric phase, samples were harvested for analysis while the remaining gastric fluids continued to the intestinal phase.

Intestinal phase

To initiate the intestinal phase, SIF was added, and pH was adjusted to 7 (± 0.1) followed by adding $CaCl_2$. Next, Pancreatin and Bile were added, and the pH was controlled and adjusted to 7 (±0.1) if needed. Incubation followed, but the duration varied between experiments (see bullet points below). Subsequently, samples were harvested for analysis. The flat end of stainless-steel lab spoons was used to harvest the remaining biofilms after digestion, to analyze if any virus remained on the coupons (for experiments one and three).

During the first two experiments, most of the biofilm on the coupons detached during the digestion model. Therefore, for the third and fourth experiments, we wanted to examine how much of the virus was still embedded in the detached biofilm as visible sediments, compared to virus amounts in the remaining digestive fluid. After the initial intestinal incubation, samples were harvested for analysis. Following the final intestinal incubation, the remaining liquid in each sample tube was transferred to 15 ml Falcon tubes. These tubes were centrifugated at 4.696 × G for five min. A visible pellet and supernatant were obtained, and 3 × 750 µl of supernatant from each sample was transferred to BBL-tubes containing 750 µl DNA/RNA Shield™. The remaining supernatant was discarded, while the remaining pellet was resuspended in 750 µl of DNA/RNA Shield™ and transferred to BBL-tubes. All other samples harvested for RNA extraction trough the digestion model consisted of 750 µl of digestive fluid transferred into BBL-tubes containing 750 µl DNA/RNA Shield™.

As a positive control (PC) to the digestion samples, three coupons from the reactor were rinsed in sterile PBS and the biofilms were scraped off as described for coupons from digestion samples.

3.4.6 Adjustments to the main protocol between the experiments

Gastric phase:

- Exp 2:
    - Samples were harvested from digestion tubes after 35 min into the gastric phase and analyzed for the virus.

Intestinal phase:

- Exp 2:
    - 35 min into the intestinal phase, the samples were harvested and analyzed for the virus.
- Exp 3 and 4:
    - After an initial incubation of 30 min, sampling was performed as described. Then, after an additional 30 min incubation (total incubation 60 min), the plastic coupons were removed while the remaining liquid was centrifuged (4.696 × G, 5 min). The pellet and supernatant were analyzed for the virus.

3.4.7 Water samples

Based on the results in the first two experiments, we wanted to investigate what happened to the virus in the influent water tank during the one-week feeding. We suspected that the virus may degrade, especially BCoV because of its enveloped properties.

Water samples of one ml were collected from the influent water container weekly, starting the second week in the third experiment and the first week in the fourth experiment (Table 1). Samples were mixed with two ml NucliSENS® Lysis Buffer and stored at -80°C until extraction. RNA-extraction was performed using the NucliSENS® miniMAG® extraction system. Extracted RNA was immediately stored on ice until used for RT-qPCR the same day.

Experiment	Time period	Adding virus	Water sampling
1	16.02. - 16.03.	Every two days Starting 16.02.	Not performed
2	23.03. - 20.04.	Weekly Starting 23.03.	Not performed
3	05.05. - 02.06.	Weekly Starting 05.05.	12.05. – 0 (n=2) 19.05. – 1 (n=2) 26.05. – 0 (n=3) 02.06. – 1 (n=3)
4	17.07. - 14.08.	Weekly Starting 17.07.	17.07. – 0 (n=3) 24.07. – 1 + 0 (n=3 + 3) 31.07. – 1 + 0 (n=3 + 3) 04.08. – 1 + 0 (n=3 + 3) 14.08. – 1 (n=3)

Table 1: Collection period and sampling points for water samples during 2023. 0; Samples taken at time zero, immediately after spiking. 1; samples taken from water after one week of incubation.

3.5 RNA-extraction

NucliSENS® miniMAG® DNA/RNA extraction kit) was used for extraction of total viral RNA from incubation experiments with sterile PP pieces, without bacteria. After the addition

of silica particles and the consequent centrifugation of samples stored in Lysis Buffer (NucliSENS®), RNA extraction was performed according to the manufacturer's instructions. The RNA was eluted in 100 µl nuclease-free water and stored in -80°C or used for RT-qPCR immediately.

RNA Miniprep Kit (ZymoBiomics™) was employed for viral RNA extraction from plastics covered with bacteria, and for digestion model samples. All BBL-tubes containing PP pieces with bacteria and virus submerged in DNA/RNA Shield™, digestion model samples and control coupon samples, were mounted on a FastPrep-24™ homogenizer (MP Biomedicals, USA) at 6 M/S. Attachment experiment samples were run for 2 × 30 seconds (s), and digestion model and control coupon samples were run at 5 × 45 s, all with five-minute breaks in between. All samples were then centrifuged at 13.000 × G for 30 s. The supernatant used for the extraction was 300 µl for attachment experiment samples and 400 µl for remaining samples. Further extraction was performed according to the manufacturer's instructions, including DNase-treatment. The final elution volume was 100 µl for attachment experiment samples, and 50 µl for remaining samples. Extracted RNA was immediately stored on ice and used for RT-qPCR the same day, or stored at -80°C.

3.6 RT-qPCR

RT-qPCR were performed on all samples from attachment and digestion model experiments. The TaqMan™ Fast Virus 1-Step Master Mix for qPCR (ThermoFisher, USA) was used, and 3 µl of RNA was added to each replicate. Negative controls (NCs, nuclease-free water) and PCs (viral RNA) were included in each run. The reactions were run in technical duplicates in an AriaMX Real-Time PCR System (Agilent®, USA). Information regarding details about primers, probes, fluorophores and cycling conditions for RT-qPCR is found in tables 2, 3 and 4.

	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'-3')	Fluorophore	References
TV	CTGGGATACCCACAACATC	GCCAGTTAACAGCTTCAGC	TGTGTGTGCCACTGGATAGCTAGCACCBHQ	FAM ROX	(Drouaz et al., 2015)
BCoV	TGGTGTCTATATTCATTTCTGCTG	GGCCACTGCCTAGGATACA	ACACGTCCCTGGCTGAAAGCTG	FAM ROX	This study

Table 2: Primers, probes, and fluorophores for TV and BCoV.

Reagent	Volume (per sample) in µl	
	TV	BCOV
4x TaqMan Fast Virus	5	5
Forward primer (10µM)	0.8	0.4
Reverse primer (10µM)	0.8	0.4
Probe (10 uM)	0.25	0.8
H_2O	10.15	10.4
RNA Template	3	3
Total	20	20

Table 3: RT-qPCR reagents and volumes.

	TV	BCoV
Reverse transcription	50℃ for 5 min	50℃ for 5 min
Initial denaturation	95℃ for 20 s	95℃ for 20 s
45 cycles	95℃ for 15 s, 55℃ for 20 s and 64℃ for 40s (data collection)	95℃ for 15 s, and 58℃ for 60 s (data collection)

Table 4: Cycling conditions for Real Time qPCR (RT-qPCR).

3.7 Droplet digital RT-PCR (RT-ddPCR) of digestion and water samples

To acquire the exact amount of viral genome copies per ml in each sample, RT-ddPCR was performed for the second and fourth experiment.

First, all RNA replicates from the same experiment were pooled together and concentrated using RNeasy® MinElute® Cleanup (Qiagen©, Germany) according to the manufacturer's instructions.

1-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad©, USA)) was used for the absolute quantification of RNA from concentrated RNA samples. The reagents and volumes used for each mixture in the RT-ddPCR are shown in Table 5. Twenty µl was transferred to cartridges (Bio-Rad©). Droplets were generated from emulsions of water-in-oil using the QX200 Droplet Generator (Bio-Rad©), according to the manufacturer's instructions. Then, the droplets were transferred to a 96-well plate, and thermal RT-PCR was performed. The ramp rate was 2 °C/sec for each step during cycling. Finally, droplets were read by a QX200 Droplet Reader (Bio-Rad©). Samples with more than 10 000 accepted droplets were used.

Reagent	Volume (per sample) in µl
Supermix	5.5
Dithiothreitol	1.1
Forward primer (50 µM)	0.396
Reverse primer (50 µM)	0.396
Probe (5 µM)	1.1
Nuclease-free water	5.808
Reverse transcriptase	2.2
RNA template	5.5
Total	22

Table 5: RT-ddPCR reagents and volumes.

Step	Time	Temp.
RT step	60 min	48 °C
Enzyme activation	10 min	95 °C
Denaturation + annealing/extension (40x)	30 sec 60 sec	95 °C 55 °C
Enzyme deactivation	10 min	98 °C
Cooling	Infinity	12 °C

Table 6: Cycling conditions for samples during RT-ddPCR. New primers and ZEN-probes with identical base sequences as written in Table 2, were ordered, and used in RT-ddPCR.

3.8 Data analysis

For RT-qPCR analysis, Agilent AriaMx (Agilent Technologies©, USA) 1.8 was used. Data was exported to Excel (Microsoft©, USA) for further analysis. The software used for RT-ddPCR analysis was QX Manager (Bio-Rad©).

The total virus copy numbers in each sample was calculated by accounting for the dilution factor of digestion samples and the sample collection of digestive fluids, throughout the digestion model. Total virus copies of the previously described input samples were acquired from RT-ddPCR, and the PC used for both RT-ddPCR and RT-qPCR. For RT-qPCR results, the total amount of virus copies from the PC was used as the initial value to calculate the total amount of virus in the remaining RT-qPCR samples. The minimum amount of virus copies required in each sample group to acquire a positive result in both the RT-ddPCR and RT-qPCR was set as the limit of detection (LOD). Due to the decreasing virus-concentrations throughout the digestion model (increasing dilution), the LOD correspondingly increases. When using LOD as a measurement, the amount of virus copies in a negative sample can range from zero to just below LOD. Calculations of virus quantities were done by using this

formula: $N_1 = N_2 \times (1 + E)^{(Ct_2 - Ct_1)}$. N_1 and N_2 respectively represent the quantity of viral RNA in the test sample and the PC sample. Ct_1 and Ct_2 correspond to the cycle thresholds for the test sample and the PC sample, respectively. The term E stands for the efficiency of the amplification process (Stoppel et al., 2023).

For virus attachment to PP pieces (both sterile and bacteria-covered), a one-tailed Mann-Whitney U test was performed to compare the amount of virus attached to each sample. Because of the small sample size, the calculation of the U-value was performed for measuring statistical significance. The critical value of U when p-value <0.05 was deemed ≤7 for all the attachments to sterile microplastic experiments. For attachment to bacteria-covered microplastics, the critical U-value for TV was deemed ≤109, and ≤42 for BCoV. To depict the variation within each sample group, the range was calculated as the difference between the highest and lowest Ct-value across biological replicates.

4 Results

4.1 Virus adhesion to microplastic

4.1.1 Attachment to sterile microplastic

Low vs high amounts of plastic

Ct-values varied more across replicates for samples with low amounts of plastic compared to samples with high amounts of plastic (larger range of Ct-values) (Table 7).

Sample	Average Ct-value	Range Ct-values	U value
L	22.90	1.27	0
H	21.78	0.27	

Table 7: Data showing the average Ct- values for virus attached to low amounts (L) and high amounts (H) of plastic. The experiment was only carried out using TV.

Time and concentration experiments

More BCoV and TV attached to the microplastics after 24 h compared to 3 h at a concentration of 10^3TCID50/ml (Table 8). There was no difference across different incubation times when the concentration was 10^4TCID$_{50}$/ml for both viruses. When the concentration increased from 10^3 to 10^4 more TV were attached to the plastic. Otherwise, no statistical significance was shown.

Virus	Sample ID	Incubation time Hours	Virus concentration, TCID$_{50}$/ml	Average Ct-value	Range Ct-values	Comparison	U-value
BCoV	3-3	3	10^3	31.72	0.79	3-3 vs. 3-4	8
	24-3	24	10^3	31.28	0.84	3-3 vs. 24-3	7
	3-4	3	10^4	31.35	1.09	3-4 vs. 24-4	9
	24-4	24	10^4	31.95	2.25	24-3 vs. 24-4	10
TV	3-3	3	10^3	28.19	3.38	3-3 vs. 3-4	0
	24-3	24	10^3	27.12	0.66	3-3 vs. 24-3	5
	3-4	3	10^4	22.90	1.27	3-4 vs. 24-4	12
	24-4	24	10^4	22.18	3.43	24-3 vs. 24-4	0
	48-4	48	10^4	22.32	2.31	24-4 vs. 48-4	16

Table 8: Data presented for each sample using the average Ct-value of three biological replicates with two technical duplicates. Sample ID: First number – incubation time in hours (three, 24 or 48). Second number –

concentration of virus solution, either 10^3TCID$_{50}$/ml or 10^4TCID$_{50}$/ml (corresponds to -3 or -4, respectively). The critical U-value is ≤7 for both viruses.

4.1.2 Attachment to bacteria-coated microplastic

The attachment of both BCoV and TV, was significantly higher to MPs covered with *A. hydrophila* than covered with *P. putida* (Table 9). Yet, no other statistical significance was observed for BCoV. For TV, attachment to microplastics coated with *L. monocytogenes* was statistically significantly higher than attachment to those covered in *P. putida*.

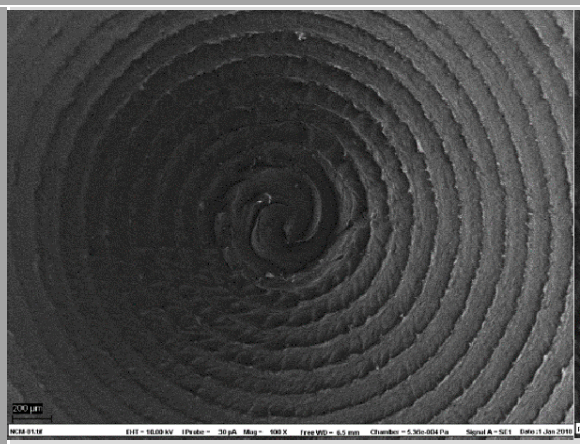
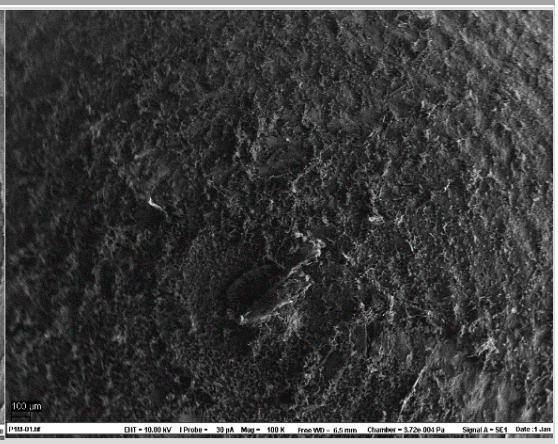
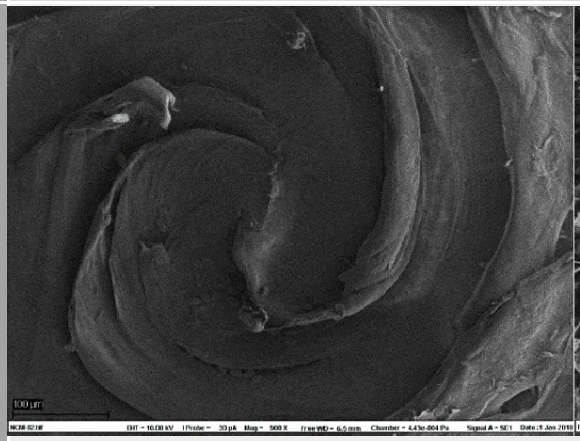
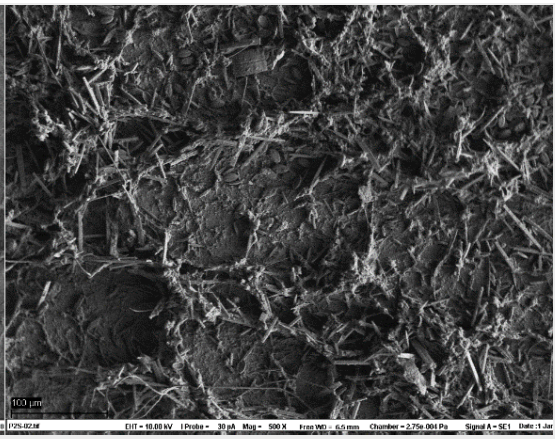
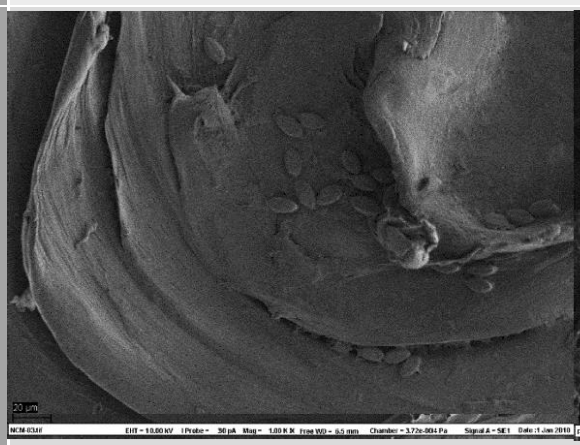
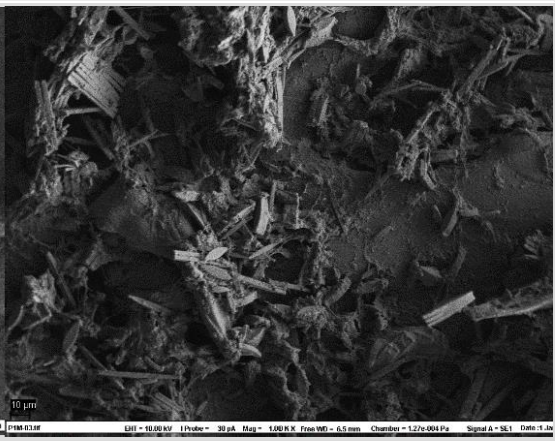
Virus	Bacteria	Average Ct-value	Range	Comparison	U-value
BCoV	*A. hydrophila* (A)	33.68	2.67	A vs. L	66
	L. monocytogenes (L)	33.56	4.25	L vs. P	47
	P. putida (P)	34.54	3.41	A vs. P	40
TV	*A. hydrophila* (A)	32.38	5.61	A vs. L	150
	L. monocytogenes (L)	31.53	2.97	L vs. P	20
	P. putida (P)	33.87	4.65	A vs. P	83.5

Table 9: Data presented using the average Ct-value of three biological replicates, and two technical duplicates per biological replicate in the RT-qPCR. The critical U-value for BCoV is ≤42 and for TV it is ≤109.

4.2 Bioreactor experiments

4.2.1 SEM

The NC coupon showed minimal biological material at 100x magnification. At higher magnifications, individual diatoms could be found towards the central areas. This was markedly different compared to the biofilm reactor coupon, which featured a multilayered biological matrix that could be seen on low magnification. On 5000x magnification, bacteria, diatoms, and other unidentified material were visualized. Nonetheless, there were also areas of the plastics that remained uncovered by this material.

Magnification	Negative control (NC)	Sample coupon with biofilm
100x		
500x		
1000x		

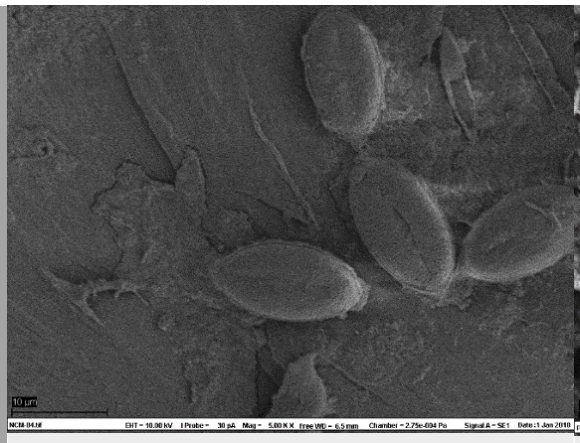
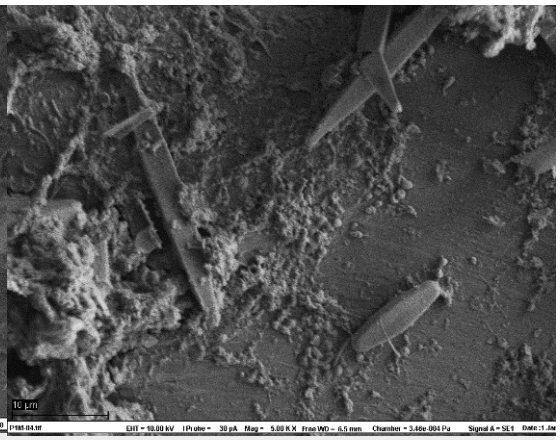
5000x		

Table 10: SEM pictures of a NC coupon after a mechanical wash and autoclaving, and a coupon with biofilm from the biofilm reactor. The coupon with biofilm was from the fourth biofilm reactor experiment where biofilms were built from 17.07.2023 to 14.08.2023. This coupon was identical to the coupons used for the corresponding digestion experiment.

4.2.2 Virus copies in total samples in the *in vitro* digestion model measured by RT-qPCR

There was more TV on the coupons going into the digestion model in the first experiment compared to the third. The opposite was the case for BCoV, where substantially higher amounts of virus were recovered from the control coupons in the third experiment.

All digestion samples were <LOD on average for BCoV in the first experiment, while this was the case for TV in the third experiment. In the third experiment, the amounts of BCoV were above LOD for all samples.

Virus	Experiment	Sample	Log_{10} Average number of viruses in total sample	Log_{10} Standard deviation	Log_{10} LOD
TV	1	Control coupons	3.33	3.08	2.19
		Gastric phase	3.88	3.73	3.20
		Intestinal phase	<3.53	3.47	3.53
		Coupons after digestion	<2.19	0.00	2.19
	3	Control coupons	2.97	2.69	2.19
		Gastric phase	<3.20	0.00	3.20
		Intestinal phase	<3.53	0.00	3.53
		Coupons after digestion	<2.19	0.00	2.19
		Supernatant	<3.53	3.25	3.53

		Pellet	<2.19	0.48	2.19
BCoV	1	Control coupons	<2.19	2.27	2.19
		Gastric phase	<3.20	0.00	3.20
		Intestinal phase	<3.53	3.25	3.53
		Coupons after digestion	<2.19	0.00	2.19
	3	Control coupons	3.87	2.90	2.19
		Gastric phase	3.53	3.56	3.20
		Intestinal phase	4.02	3.88	3.53
		Coupons after digestion	3.51	3.34	2.19
		Supernatant	4.65	4.44	3.53
		Pellet	3.72	2.31	2.19

Table 11: RT-qPCR was used to measure the total amount of virus copies in each sample. Ct-values were used to calculate the total amount of virus copies in each sample. Data is presented as average across biological replicates. The limit of detection (LOD) was determined by accounting for the continuous dilution of digestion samples throughout the digestion model (decreasing virus concentration), resulting in a larger amount of virus required in each sample to obtain detection in RT-qPCR. Some samples are labeled as "below LOD" (<LOD), meaning that the total amount of virus in the sample is below what is required for a positive result in the RT-qPCR. Noticeably, some samples labeled <LOD have standard deviations above zero. This is because some replicates within these sample groups are above LOD, even though the average virus amount across technical and biological replicates is <LOD.

4.2.3 Total number of virus copies of the *in vitro* digestion model samples measured by RT-ddPCR

In the second experiment, control coupons were above LOD for both viruses, with higher amounts of TV compared to BCoV. The samples from the intestinal phase were above LOD for TV, indicating that most of the virus in the biofilm had fallen off the coupons during the gastric phase. For BCoV, both gastric and intestinal samples were below LOD, which coincides with low amounts of the virus on the control coupons.

In the fourth experiment, there was a substantial difference in the amount of virus that was recovered from the control coupons. It appears all the TV detached from the coupons during the gastric stage. This accounts for BCoV as well, but additionally, some virus was recovered in the supernatant after digestion and centrifugation. TV reappears in the pellet after finished

digestion, while it was not found in the supernatant, suggesting that it remained incorporated in the biofilm through digestion.

Virus	Experiment	Sample	Log_{10} Total number of virus copies	Log_{10} LOD
TV	2	Control coupons	1.51	0.48
		Gastric phase	<1.41	1.41
		Intestinal phase	1.80	1.75
	4	Control coupons	4.07	0.48
		Gastric phase	4.10	1.41
		Intestinal phase	<1.75	1.75
		Supernatant	<1.75	1.75
		Pellet	2.27	0.48
BCoV	2	Control coupons	1.38	0.48
		Gastric phase	<1.41	1.41
		Intestinal phase	<1.75	1.75
	4	Control coupons	1.94	0.48
		Gastric phase	2.52	1.41
		Intestinal phase	<1.75	1.75
		Supernatant	2.58	1.75
		Pellet	<0.48	0.48

Table 12: Using RT-ddPCR, all biological replicates were pooled and analyzed for total virus amount. Data is presented as the total amount of virus in each sample group. The limit of detection (LOD) was determined by accounting for the continuous dilution of digestion samples throughout the digestion model (decreasing virus concentration), resulting in a larger amount of virus required in each sample to obtain detection in RT-ddPCR.

4.2.4 Virus concentrations in spiked river water

Experiment 3

The influent water was spiked with a more consistent and larger amount of TV than BCoV. During the first week of sampling, the degradation of TV was lower compared to BCoV. Over

the last week of sampling, the degradation of TV was higher compared to BCoV, considering the difference in the amounts between the viruses.

Virus	Date of sampling for virus quantification	Sample	Log_{10} Virus copies/ml	Log_{10} Virus reduction after one week
TV	12.05.	Fresh water	5.90	1.90
	19.05.	One week old water	4.00	
	26.05.	Fresh water	5.95	4.47
	02.06.	One week old water	1.48	
BCoV	12.05.	Fresh water	4.92	3.04
	19.05.	One week old water	1.88	
	26.05.	Fresh water	5.41	3.03
	02.06.	One week old water	1.48	

Table 13: The third biofilm reactor experiment started 05.05.2023, and fresh water was spiked with BCoV and TV. The first water samples were collected after the second time of adding fresh water spiked with virus, i.e., one week after starting the experiment. After the experiment's second week, only samples of one week old water were collected. On week three, samples were again collected exclusively from fresh water, and after the fourth week, from one week old water. Data are presented as the average of two biological replicates on 12.05. and 19.05., and three biological replicates on 26.05 and 02.06. LOD in the RT-ddPCR was 28.

Experiment 4

The amount of TV added to the influent water tank weekly was more than double that of BCoV. After one week of incubation, the amount of virus was consistently lower for BCoV compared to TV, except for the last week of sampling.

Virus	Date of sampling for virus quantification	Sample	Log_{10} Virus copies/ml	Log_{10} Virus reduction after one week
TV	17.07	Fresh water	5.80	2.53
	24.07.	One week old water	3.27	
	24.07.	Fresh water	5.95	1.82
	31.07.	One week old water	4.13	
	31.07.	Fresh water	5.79	1.78
	07.08.	One week old water	4.01	
	07.08.	Fresh water	5.78	2.00

	14.08.	One week old water	3.78	
BCoV	17.07.	Fresh water	5.52	4.07
	24.07.	One week old water	1.45	
	24.07.	Fresh water	5.53	4.08
	31.07.	One week old water	1.45	
	31.07.	Fresh water	5.48	
	07.08.	One week old water	1.45	4.04
	07.08.	Fresh water	5.30	2.88
	14.08.	One week old water	2.42	

Table 14: The experiment started 17.07.2023, where the first water samples were harvested. The water samples were harvested weekly before and after adding fresh water and virus. The last day of sampling was 14.08.2023. Data presented as the total amount of virus copies for all samples taken on the given date. LOD for all samples in RT-ddPCR was 28.

5 Discussion

5.1 Attachment of virus to sterile microplastic

The purpose of the attachment experiments was to investigate if the chosen viruses were able to attach to the plastic type we were going to use further on in the project, and to find if the viruses attached these plastics after incubation with different bacteria.

Even though statistical significance was shown between samples incubated for 3 h in different concentrations of TV and the attachment of TV after 3 h compared to 24 h, we regarded the attachment after 3 h using a concentration of about 1.0×10^3 $TCID_{50}$/ml as sufficient for further experiments. Oppositely for BCoV, only incubation time affected the attachment to plastics and not the increased concentration. Since the effect of incubation time on attachment was relatively small, and to make experiments easier, we continued further work with the same incubation times and concentrations for BCoV as for TV.

A possible explanation for the abovementioned differences in attachment could be that since TV is smaller in size than BCoV, there is more space left for attachment to the plastic for TV than BCoV. Additionally, it is important to note that when calculating the titer of a virus stock by using $TCID_{50}$/ml, only the infective viruses are considered (Lei et al., 2021). This means that we do not have control over the non-infective viruses in the stock virus solution. Even though they are not infectious, these viruses can still attach to surfaces and in the end contribute to the RT-qPCR result as long as their RNA is undamaged (Gassilloud et al., 2003).

If the amount of non-infective viruses is considerably higher in the TV solution than in the BCoV solution, this could affect the results by contributing to misleadingly low Ct-values for TV samples compared to BCoV samples, even at seemingly equal concentrations.

Lastly, two repetitions of these experiments would have made the results more reliable and trustworthy. However, since these experiments were preliminary studies, we concluded we had collected enough information.

5.2 Attachment of virus to bacteria-coated microplastic

Interestingly, both viruses attached better to MPs incubated with *A. hydrophila* than those incubated with *P. putida*. Also, TV attached better to *L. monocytogenes*-microplastics than *P. putida*-microplastics. There are numerous ways bacteria and different viruses may interact (Gagné et al., 2022). Potentially, TV surface proteins more easily bind to other microorganisms' surface structures such as lipopolysaccharides, polysaccharides, peptidoglycans, and HBGA-like carbohydrates, than BCoV surface proteins (Amarasiri & Sano, 2019; Robinson et al., 2014). Additionally, HuNoV, the virus for which TV is a model virus, has been proven to bind to both pathogenic and commensal bacteria which express different HBGA-like carbohydrates on their surface (Li et al., 2015). Furthermore, a study by Miura et al. demonstrated through transmission electron microscopy (TEM) that different *Enterobacter* species interact with virus-like particles (VLPs) of HuNoV, and these VLPs primarily bind to the extra polymeric substances (EPS) where HBGAs are present (Miura et al., 2013). It has also been found that poliovirus (naked virus) binds bacterial surface polysaccharides, which resulted in enhanced environmental stability including more resilience towards heat and chlorine treatment (Robinson et al., 2014). Hence, there is a possibility that TV binding to surface proteins and HBGAs promotes the virus' environmental fitness, and more so compared to BCoV.

Lastly, SEM pictures, taken by another research track student, of biofilm under equal incubation conditions, show that large areas of the plastic surface are not covered by bacteria. Therefore, it could be that the type of bacteria is irrelevant. Considering that both viruses attached well to sterile microplastics, there is a possibility that the viruses attach to the remaining, non-bacteria-coated plastic surface, rather than to the bacteria. As stated in the introduction, the isoelectric point (IEP) of viruses plays a part in attachment to different cells (Dika et al., 2015). The IEP of TV is 10, while it is about 4.6 for BCoV (Farkas et al., 2008; Kapil et al., 1999). However, other factors like the charge and total size of their capsid and

RNA/DNA also affect the attachment (Dika et al., 2015). In addition to IEP and the total charge of the viruses, factors like hydrophobic forces, pH of the surroundings, non-specific electrostatic interactions, surface roughness, and surface charge of the plastic surface are important to assess the total amount of forces leading towards or from adhesion of a surface (Dika et al., 2015; Dika et al., 2013). Because of TV's and BCoV's contrasting IEP, they will behave differently according to the pH in their surroundings (Gassilloud & Gantzer, 2005). Combined, these factors explain the complexity of the adhesion interactions of the viruses to plastics and other surfaces.

For attachment to bacteria-coated microplastics, the experiment was repeated two times for TV but only one time for BCoV. Ideally, we would have repeated the BCoV experiments two times, but because of time shortage we regarded it as a sufficient repetition to get an indication of the trend with only one repetition (two experiments).

5.3 Biofilm reactor experiments

5.3.1 SEM

The coupons were reused and exposed to mechanical cleaning and autoclaving after each experiment. Nevertheless, the SEM pictures show organisms present on the NCs indicating that mechanical cleaning with a dish brush and dish soap, followed by autoclaving, was not enough to remove all biological materials between each experiment. The organisms found on the NCs are probably a form of microalgae called diatoms (Blenkinsopp & Lock, 1994; Jiang et al., 2015).

The NC pictures show the microstructure of the plastic coupons. There are lots of cracks and crevices where microorganisms easily can attach and nudge themselves.

The biological material on the sample coupons appears complex with numerous different organic compounds, and diatoms are also found on the biofilm-covered coupons (Blenkinsopp & Lock, 1994; Jiang et al., 2015).

5.3.2 Biofilms, water, and environmental conditions

Biofilm formation highly depends on the surrounding environment, especially factors such as available nutrients and temperature (Holá et al., 2006; Villanueva et al., 2011). In general, warmer water temperatures compared to colder, favor the growth of microorganisms and the formation of biofilms (Bell et al., 2021; Villanueva et al., 2011). Temperatures in the river water, water flow and precipitation in the proximity of the river (runoff), thereby affect the growth of different types of microorganisms in the river water. However, the bacterial

community making up the plastisphere seems to overlap during different times of the year, where bacteria from the Proteobacteria phylum seem to be the most abundant regardless of season (Witsø et al., 2023). It is reasonable to assume that if there was an increased number of bacteria entering the biofilm reactor, this would have resulted in more bacteria present in the biofilm during the warmer months. Regarding viruses in biofilms, a study by Gagné et al. showed significant increases in the attachment of rotavirus and HAV to surfaces colonized by different types of biofilms compared to non-colonized surfaces, but not for Murine Norovirus (HuNoV model virus) (Gagné et al., 2022). They also discuss that different viruses behave differently in biofilms, which our results highly support.

Results from the first, second and fourth biofilm reactor experiments could indicate that TV is more easily incorporated into the biofilm than BCoV. However, since the amounts of virus in the biofilms on the coupons during the two first experiments were low, we wanted to investigate what happened to the viruses in the influent water during incubation in the two following experiments (3 and 4). The detected amount of TV is notably higher than the amount of BCoV in both the third and fourth experiments. This could account for some of these differences in the biofilm incorporation of the viruses. Reasons for this could be that TV is more resistant to degradation than BCoV, and in general, non-enveloped viruses are more resilient than enveloped viruses (discussed later).

Interestingly, in the third experiment, the incorporation of BCoV in the biofilm on the control coupons is notably higher than that of TV, despite the lower input amount of BCoV in the influent water tank. However, TV detection from the influent water tank during the last week of incubation in this experiment indicates that the virus is heavily degraded, and even more so than BCoV. One could speculate that at this point, conditions may have favored BCoV more than TV.

Factors such as elevated temperature, UV radiation, and microbiological activity can contribute to the degradation process (Ahmed et al., 2020). Research has shown that non-enveloped viruses in surface water are more degraded than in distilled water, but less degraded than such viruses in wastewater, even at identical temperatures. This implies that the physiochemical properties of their surroundings greatly impact their infectivity, and support our findings that the viruses in the influent water are rapidly degraded, especially BCoV (Casanova & Weaver, 2015). In addition, both the influent water tank and the biofilm reactor were exposed to sunlight through the laboratory windows, potentially influencing the results.

To mitigate this, the influent water tank and the reactor could have been shielded with a light-reflective material to prevent UV radiation from reaching the samples. However, it is important to note that UV radiation is a natural occurrence in river environments, thus its presence could arguably enhance the ecological validity of the experiments by making them more representative of natural conditions. UV-B- and UV-C-waves are almost entirely blocked by glass, while UV-A-waves are not. For disinfection, UV-C is the most used, while UV-A does not cause the same amount of microbiological damage (Gray, 2014). Therefore, it is not likely that UV radiation would be an important confounding factor in our research, but it is possible that the sunlight contributed to increasing the water temperature. The water temperature was only measured once, and at that point, the water temperature was equal to RT.

5.3.3 In vitro digestion model

We noticed that in the first two experiments, the biofilm quickly detached from the PP coupons already in the gastric phase. Consequently, the question we needed to address was not whether the viruses were more protected when associated with the plastisphere or plastic surface (since the plastic was no longer involved) but rather if the detached biofilm itself protected the viruses. Therefore, we decided to implement centrifugation of digestion samples after digestion was finished for the third and fourth experiments, to be able to analyze the detached biofilm. After centrifugation, visible pellets with biofilm material were obtained. The pellets were BCoV positive in the third experiment, while they were TV positive in the fourth. Since we got positive results, we can speculate if this means that the viruses were protected from degradation during digestion. However, because of the dilution factor during digestion, we must also consider that a smaller amount of virus was required to acquire a positive result in either the RT-qPCR or RT-ddPCR when the virus was in a solid sample compared to a liquid sample. This means that it could have been at least the same amounts of virus in the other samples, but because of the digestion model's dilutional effect on virus concentration in each sample, these samples were instead below LOD. In addition, if we observe both the LOD and the amount of virus in the intestinal phase samples, it seemingly increases. This is most likely because we were not able to scrape off all the viruses from the control coupons, but in digestion, all the material detached (this matter is discussed later).

Experiment 1

Results from control coupons in this experiment show that the amount of TV on the coupons that go into the digestion is above LOD, increasing the probability of the virus being detected in the gastric phase. For BCoV, the control coupons were negative for the virus on average, except for two technical duplicates from different samples that were above LOD. The negative results for both viruses during the intestinal phase are most likely due to the probable simultaneous virus and biofilm detachment from the coupons during the gastric phase (as most biofilm detached at this stage), combined with the increased dilution of digestion samples from the gastric to the intestinal phase. In addition, the negative results from scraping of the coupons after digestion coincide with the observed detachment of biofilm.

Experiment 2

In this experiment, both viruses were detected in the control coupons. Interestingly, no virus was recovered from the gastric phase for either virus, but TV reappears in the intestinal phase. A reason for this could be that the biofilm did not completely detach from the coupons during the gastric phase, but rather in the intestinal phase. It could also be that the prolonged presence in the digestion model broke down the biofilm, perhaps making it easier to extract RNA from the material. However, the extraction kit that was used (ZymoBiomics™ RNA Miniprep Kit), is designed to deal with complex environmental samples, so this should not be the case.

Experiment 3

Much more BCoV than TV was detected on PC coupons in this experiment. For TV on average all the samples from the digestion were below LOD. On the contrary, BCoV was detected in almost all samples throughout digestion, where the average number of viruses was well above LOD. Thereby, the results from this experiment differ from the other digestion experiments. In this experiment there was visible biofilm on the coupons after the completion of the digestion model. Since no obvious changes were made during this experiment that could explain this difference, the reason for some biofilm remaining on the coupons could be in its composition. The composition of biofilms can be affected by numerous factors, such as available nutrients, physical shear forces, temperature, quorum sensing and different productions of transcription factors by different bacteria (Stanley & Lazazzera, 2004). In our case, physical shear forces are the only factor we have control of, because of the repeated use of the identical RPM across biofilm reactor experiments. In addition, the temperature is likely to be identical if the room temperature was stable.

Experiment 4

Here, TV was found in abundance on control coupons, while a lesser amount of BCoV was found. Again, one can only speculate as to why this happens, but as discussed previously, it is hard to find the exact underlying mechanisms. The high amounts of TV in the gastric phase suggest that most of the virus has detached from the coupons. Interestingly, BCoV is found again in the supernatant, whilst TV is not.

5.4 Exploring the use of infection assays

A cell-infection experiment was performed with means to investigate how the viruses' infectivity was affected by passing through the *in vitro* digestion model. In addition, we wanted to know how the digestive fluid itself would affect the cells, and if it would be possible to use cell-lines for further infectivity experiments. We decided to exclude this from Materials and Methods because it was a single pilot experiment. Results proved that working with the digestion model, viruses and cell lines proved to be challenging since the digestive fluid was toxic to the two cell lines used for growing the viruses, even at 10^{-3} dilutions. This is most likely because of the enzymatic activity in the digestive fluid. Pepsin and trypsin can degrade cell surface proteins, lipases may break down fats in the cell membranes, and bile salts can disrupt cell membranes (Bathoorn et al., 2011; Mustranta et al., 1995; Sagawa et al., 1993; Sutradhar et al., 2010).

5.5 Enveloped vs. non-enveloped viruses

In our research, the general trend has been that the enveloped virus BCoV is less stable and resilient than the non-enveloped TV, which is what we expected to happen beforehand. This could be because in general, enveloped viruses are more vulnerable to degradation stressors such as dehydration, chemical disinfectants, soaps, and high temperatures, than non-enveloped viruses (Wood & Payne, 1998). The answer to this is in the structure of the envelope, which consists of a bilayer of lipids with surface proteins. Functions pertaining to infection and entry into cells, are carried out by the envelope with its surface proteins. That means, when the envelope is damaged, the virus becomes non-infective. Since the lipid bilayer is more easily disrupted and degradable than the virus capsid, which mostly consists of proteins, enveloped viruses more easily lose their infectivity in the environment than non-enveloped viruses (Casanova & Weaver, 2015; Wood & Payne, 1998).

5.6 Extrapolation possibilities

Many of today's widespread viral infections are caused by enveloped viruses, such as influenza viruses, respiratory syncytial virus, HIV, Ebola virus and Nipah virus (Rey & Lok, 2018). One could argue that because BCoV is an enveloped virus, the findings from our work may be extrapolated and applied to the wide variety of enveloped viruses. For example, the HE of BCoV bears similarity to the hemagglutinin-esterase-fusion glycoprotein of the influenza C virus, indicating they might share some common functionalities (Saif & Jung, 2020; Wang & Veit, 2015). On the other hand, the structure and hardiness of different enveloped viruses vary greatly. Even though they show fundamental likeness across both genus and species, other properties greatly impact their persistence, viability, and infectivity.

If we would have liked to extrapolate the results to all enveloped viruses, the bacteriophage Phi6 might have been more suited than BCoV, as it is widely used as an enveloped virus model virus (Aquino de Carvalho et al., 2017; Moresco et al., 2022). While model viruses are useful, they often provide less scientific value than studying the virus of interest. This is because the model viruses rarely share all the same properties as the "actual" virus.

5.7 Methodological considerations

5.7.1 To biofilm or not to biofilm

The incubation with bacteria and plastics lasted for approximately 18 hours. When looking at SEM pictures of the plastic pieces after this incubation, it was clear that there was not a proper biofilm formation with these samples under the provided conditions. The bacteria attached to the plastic is mostly a singular layer, and far from covering the entire surface of the plastic. Most likely, a complete biofilm would have formed if the samples were left for further incubation under appropriate conditions. Therefore, we refer to this as bacterial covering or coating of microplastics rather than biofilm formation on microplastics. To be clear, this does not account for the plastic coupons in the biofilm reactor. Here, proper biofilms were formed.

The biofilm on some coupons was quantified using the standard biofilm quantification method with crystal violet staining and measuring the absorbed color. However, this was not done routinely. This could have been valuable information to examine the variation in biofilm formation across the different experiments, and it would have provided more solid scientific proof of the observations made during the experiments. For instance, we observed that there

was much more material on the coupons compared to the other digestion experiments when starting digestion in the third experiment, but we have no data to strengthen this claim.

5.7.2 Size of plastic

The size of the plastic pieces used in attachment experiments was not uniform. Even though the weight was approximately identical, the surface-to-volume ratio differed between plastic pieces. Ideally, we would have bought and used completely spherical and identical PP pieces instead of the pieces we used. That would have made the experiments more identical and probably more reliable.

The plastic coupons we used in the *in vitro* digestion model are unrealistically large for humans to consume. However, this experiment does not try to provide information on what happens in the human digestive system when exposed to plastispheres. Rather, we try to provide basic information regarding the attachment of a biofilm and viruses in this biofilm, through an artificial digestion model. In addition, we needed a large surface area to build enough biofilm on the coupons using the river water. Even after four weeks of incubation, the biofilm growth was modest, and the amount of virus in the biofilm was relatively low compared to the amount of virus that was inoculated in the influent water.

5.7.3 SEM

Because of the apparent low quality of the pictures when samples first were coated with 15 nm of platinum, they were coated one more time simultaneously with the NC. However, this did not improve the picture quality noticeably.

An alternative imaging method to picture the viruses would have been to use TEM, which is the single method for imaging viruses (Roingeard et al., 2019). However, after discussions with the imaging engineers at NMBU, these samples were deemed unfit for that technique.

5.7.4 Use of the *in vitro* digestion model

In line with the 3R's (replace, reduce, refine), every researcher should seek to minimize their use of research animals (National Centre for the Replacement, 2023). In addition to the ethical considerations, research on animals is also time consuming, complex, and expensive, and the experiments become easier to repeat when there is significantly less biological variation.

Digestion physiology is complicated, and a static *in vitro* digestion model oversimplifies the digestive process, for example by not considering the dynamic nature of digestion and the omission of gut microbiota in the samples. However, there are not enough solid arguments to

defend the use of research animals in my studies, even though that may reduce the practical implications. Therefore, we have used an *in vitro* digestion model that mimics the human digestive system, developed by the INFOGEST community in 2014, to study viruses and the plastispheres in human digestion. There have been several *in vitro* digestion protocols described, but this is a universal protocol for static *in vitro* digestion model which provides increased repeatability across studies and laboratories (Brodkorb et al., 2019; Mackie et al., 2020). The model is predominantly designed for digestion experiments regarding the digestibility of protein, lipids, starch etc. in different foodstuffs, and not for microbiology experiments (Brodkorb et al., 2019). However, two former PhD-students in the same department at the university have used this model in their doctoral work studying *L. monocytogenes and* extended-spectrum beta-lactamase resistant *E. coli* (Buberg et al., 2023; Pettersen et al., 2019). However, they did not work with biofilm, but with monocultures of bacteria.

According to the original INFOGEST *in vitro* digestion model protocol, it is ideal to have at least three replicates for each digestion step. This approach prevents the need to sample from the same tube across multiple digestion phases (Brodkorb et al., 2019). It would also have simplified the process of calculating the virus amount in each sample, since the combination of increasing dilution with a changing volume due to sampling, makes this calculation complicated. To meet this requirement, we would need at least two additional biofilm reactors.

5.7.5 Scraping of coupons after digestion and biofilm-detachment method

Before the first digestion experiment, we wanted to examine if there could be viral particles stuck to the plastic after digestion, so the coupons were scraped even though there was no visible biofilm left. Because of the negative results, coupons from the second and fourth experiment which did not have visible material left after digestion, were not scraped. However, there was material left on the coupons after the third digestion experiment, so they were also scraped.

As briefly mentioned previously, it can seem like the total amount of virus in the samples that have passed through the digestion model is larger than the amount from the control coupons. This is most likely due to the procedure used to detach biofilm from these coupons. To make sure to get every little bit of virus from the control coupons, the method should have been more thorough than the mechanical scraping of the coupons into BBL-tubes. One solution

would be to soak or shake the coupons in the volume of DNA/RNA Shield® used further for extraction. By doing this, we would have achieved a more precise picture of the virus amount in the biofilms. Nevertheless, we considered this unnecessary, since good results had been achieved by scraping biofilm off plastic in a separate work package within the same project (Witsø et al., 2023). However, the effectiveness of this method was unknown.

5.7.6 Choice of RNA extraction kit

RNA extraction of samples consisting of microplastic incubated with virus alone was performed using the NucliSENS® miniMAG® extraction kit. However, in one of the first experiments using bacteria-coated microplastics, the silica particles that bind the RNA during extraction, aggregated instead of mixing homogeneously. The corresponding RT-qPCR results also showed some negative results and more irregular Ct-values. After a discussion, we figured that the most probable cause of this was the bacteria in the samples and that the extraction kit was not made for these types of "contaminated" samples. Therefore, the RNA Miniprep Kit (ZymoBiomics™) was employed for RNA extraction in the following experiments with bacteria. This is a kit designed for environmental samples.

We have also reflected on the inconsistency in RT-qPCR results, especially regarding the digestion model samples which often had large variations of Ct-values.

RT-qPCR inhibitors or RNases could have been present during the extraction. RNases, a form of nuclease, exist ubiquitously and break down RNA. Therefore, RNA extraction should be conducted in a nearly sterile environment (Green & Sambrook, 2019). Since the DNA/RNA Shield™ provided by the extraction kit should inactivate nucleases in the sample, any nucleases must have been introduced later in the extraction process, for example via contaminated pipettes, pipette tips, and extraction reagents. I would point at the extraction reagents and the pipette tips used during extraction. When performing RNA extraction, producers recommend using filtered, certified nuclease-free pipette tips. This was performed with all samples extracted with the nucliSENS® miniMAG® kit since extraction was carried out in the virology lab, where filtered pipettes are routinely used. However, when we extracted RNA with the ZymoBiomics® kit, we used ordinary autoclaved pipette tips due to the routine use in the food safety lab. Therefore, it is valuable to note that autoclaving itself is not proven to remove RNases (Green & Sambrook, 2019). In addition, extraction reagents were used for several extractions. Since the bottles were continuously re-opened and closed, resulting in more exposure to the environment, this probably increased the risk of

contamination with nucleases. To summarize, there is a possibility that at least some parts of the varying results are due to nuclease-contamination.

For the samples from the digestion model, we suspected that the differences in pH (pH3 and pH7) could be the reason for varying results. To exclude this, we performed an experiment with digestion samples before and after the adjustment of pH after the gastric phase. Here, samples were taken right after the gastric phase at pH 3 (+-0.1) and right after adjustment to pH 7 (+-0.1). The results showed no difference between these samples.

5.7.7 PCR and RNA considerations

RT-qPCR is a poor method to study infectiousness, as it can detect viral RNA from both infectious and non-infectious virus particles (Duizer et al., 2004; Tian et al., 2013). Since working with $TCID_{50}$ infectivity assays proved to be too challenging (as previously discussed), we also considered incorporating PMaxx™ pre-treatment of samples to differentiate between infectious and non-infectious viruses. This pre-treatment has been proven to be more effective in distinguishing between non-infective and infective virus particles than conventional RT-qPCR (Randazzo et al., 2018; Razafimahefa et al., 2021). The PMAxx™ dye penetrates damaged virus capsids and binds to RNA, which reduces amplification and signaling in following RT-qPCR. However, we deemed this method too complicated for use in this project for several reasons. For one, since the pre-treatment is dependent on a photo-activating system, it would have been challenging to illuminate the whole sample equally when using the small plastic beads. Therefore, we also debated if we could implement the use of RNase as a pre-treatment instead (Monteiro & Santos, 2018). However, the virus concentration in most of these samples was too low to yield a positive result on RT-qPCR, even without the pre-treatment. Therefore, carrying out these pre-treatments would not have yielded meaningful results.

RT-ddPCR was only run on samples from experiments two and four. This was because the RNA from the first experiment was used in both a RT-qPCR run and was up-concentrated for a second RT-qPCR run, using all of the RNA. The samples from the third experiment were not included in the RT-ddPCR run due to a misunderstanding. Because of this, we regarded it as sufficient to use the total number of virus copies acquired through RT-ddPCR, for the PC used in all RT-qPCR runs and use that number as a base to calculate total virus copies (as described earlier).

5.7.8 Documentation of environmental conditions

In research, there will always exist a conflict between "natural" conditions and repeatability and reproducibility. Repeatability and reproducibility of experiments are foundation stones in modern science, to ensure the validity and precision of scientific results and to prevent bias. How repeatable and reproducible an experiment is, would in many cases negatively correlate to the potential confounding factors one may find under "natural" conditions. To combat this, the "natural" conditions should be well documented. In our case, this would have meant for example measuring the water temperature in both the influent water tank and the biofilm reactor constantly, analyzing every new batch of river water for conductivity, pH, minerals and so forth, and measuring the UV radiation coming from the sun through the windows in the lab. Frankly, the measuring of all these parameters was seen as too resource intensive.

5.7.9 Variation of results and Future research

Furthermore, the large variation in results needs to be addressed. Working with environmental samples, as we have previously discussed, involves many unknown variables. It is quite different from executing standardized experiments in the lab, where conditions can be kept consistent across experiments in a study. For those who would wish to perform a similar study, we have a couple of suggestions:

1. Even though the apparent low amounts of biofilm after two weeks, it can be possible to reduce incubation time from four to two weeks. This is because of a potentially high turnover of biofilm and continuous detachment and attachment of viruses to these biofilms, so four weeks of incubation probably do not result in a higher virus yield in the biofilms than two weeks of incubation. Therefore, it is possible to perform more repetitions by reducing the incubation period.

2. Instead of building biofilm by using river water, cultures of already known bacteria can be used for each experiment. Even though this would probably have produced more uniform results and given better answers to whether the viruses are protected by the biofilm through the digestion model or not, such an experiment would have been more distant to "real-world" applications.

Moreover, even though the detection of viral RNA is not synonymous with infectious viruses, some of the positive results might stem from infectious viruses. Since we have found viral RNA in all phases of the *in vitro* digestion model, we might have moved an inch closer to gaining an answer for what complications ingestion of plastispheres may have for human

health. The next important step would be to assess the infectious potential of these viruses when embedded in plastispheres compared to when they are not, after being exposed to the *in vitro* digestion model.

6 Conclusive remarks

To start answering our initial hypothesis about virus attachment to plastics, our results have shown that both TV and BCoV were able to attach to both sterile and bacteria-coated MPs after 3 h in a virus concentration of about 1.0 x 10^3 $TCID_{50}$/ml.

When studying our results regarding the incorporation of viruses in environmental biofilms (plastispheres), and their stability through the *in vitro* digestion model, they both support and reject previous claims about enveloped viruses being more degraded in plastispheres than non-enveloped viruses. More TV is detected in the first, second and fourth experiments, while BCoV is detected to a much higher degree than TV in the third experiment. Therefore, in this case, it could be that biofilm facilitates viral survival, and it tells us that different environmental factors probably favor the survival of different viruses. However, there is a clear trend that TV is more resilient both when incorporated in a plastisphere and in river water, partially confirming our hypothesis that TV is more resilient than BCoV.

Furthermore, it seems that whenever the viruses are abundant in the biofilms on the control coupons, they are also detected further on in the digestion. Apart from that, it is hard to depict a clear trend in which virus persists the best throughout the digestion model. Ideally, the third experiment in combination with the water sampling for the fourth biofilm reactor experiment, would have been the protocol from the start. In conclusion, we can certainly state that working with environmental samples with ever-changing characteristics is challenging.

My work has generally consisted of exploring a method, doing several pilot studies, and investigating how it is to combine work with the INFOGEST *in vitro* digestion model with plastic, biofilm, and viruses. No one in the department had combined experiments with viruses, bacteria, and plastic before. Moreover, we have not found any literature where investigation of, and experiments with, viruses incorporated in plastispheres and their destiny in human digestion models, have been conducted. Therefore, even though our results vary, we view our work as valuable contributions to the body of knowledge in microbial, virological and food safety fields.

Nonetheless, a key lesson I have learned during my research track work is that regardless of the plans and assumptions we can make, unforeseen circumstances will arise and disrupt

them. There has been a lot of “could have, should have”, but this has in hindsight made me more analytical and aware of everything one must consider when conducting research. So, to endthings off, we can ponder over these words by the great poet Antonio Machado “Traveler, there is no path. The path is made by walking”.

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