OXYVIR 2 project

Control of norovirus hazard in bivalve molluscan shellfish and their environment

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31 may 2023

Aquaculture Advisory Council (Brussels)

Norovirus: classification and importance

- **Human norovirus (NoV)**: ~ 20% of all acute gastroenteritis worldwide
- Replication of NoV only in humans for some specific genogroups
- Small particle (~ **40 nm**) with a **RNA** genome (~ 7.5 kb)
- Low ID_{50}: **~10-100 particles** by analogy to other enteric viruses
- Shedding in the environment (stools, vomits) : ~ **10^6 to 10^9 particles/g**
- **Naked virus:** more resistant than enveloped viruses in the environment

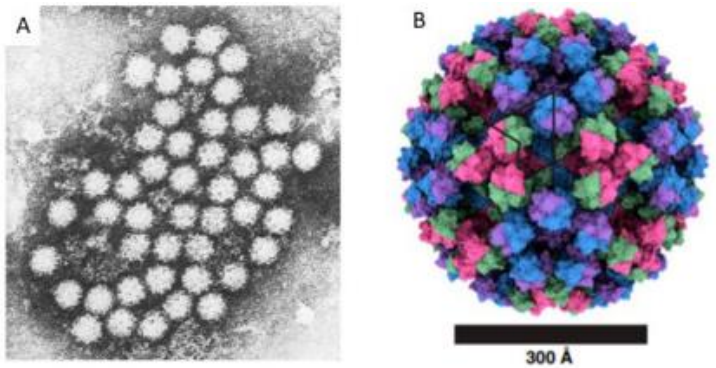
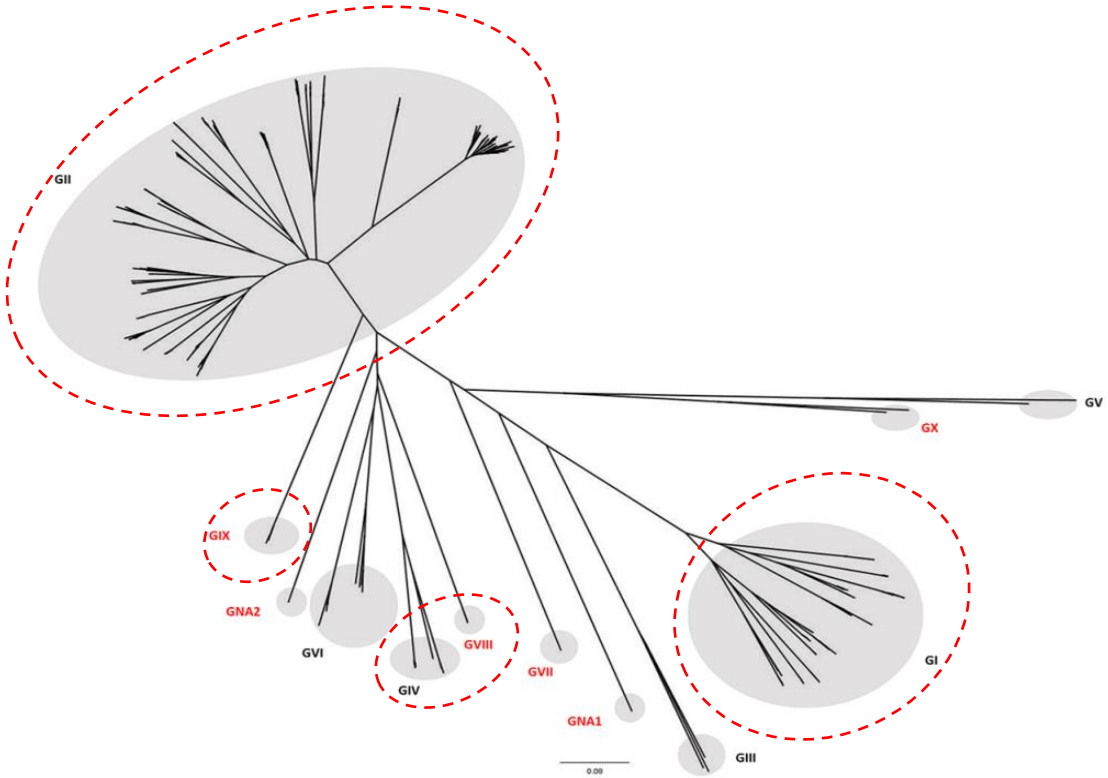


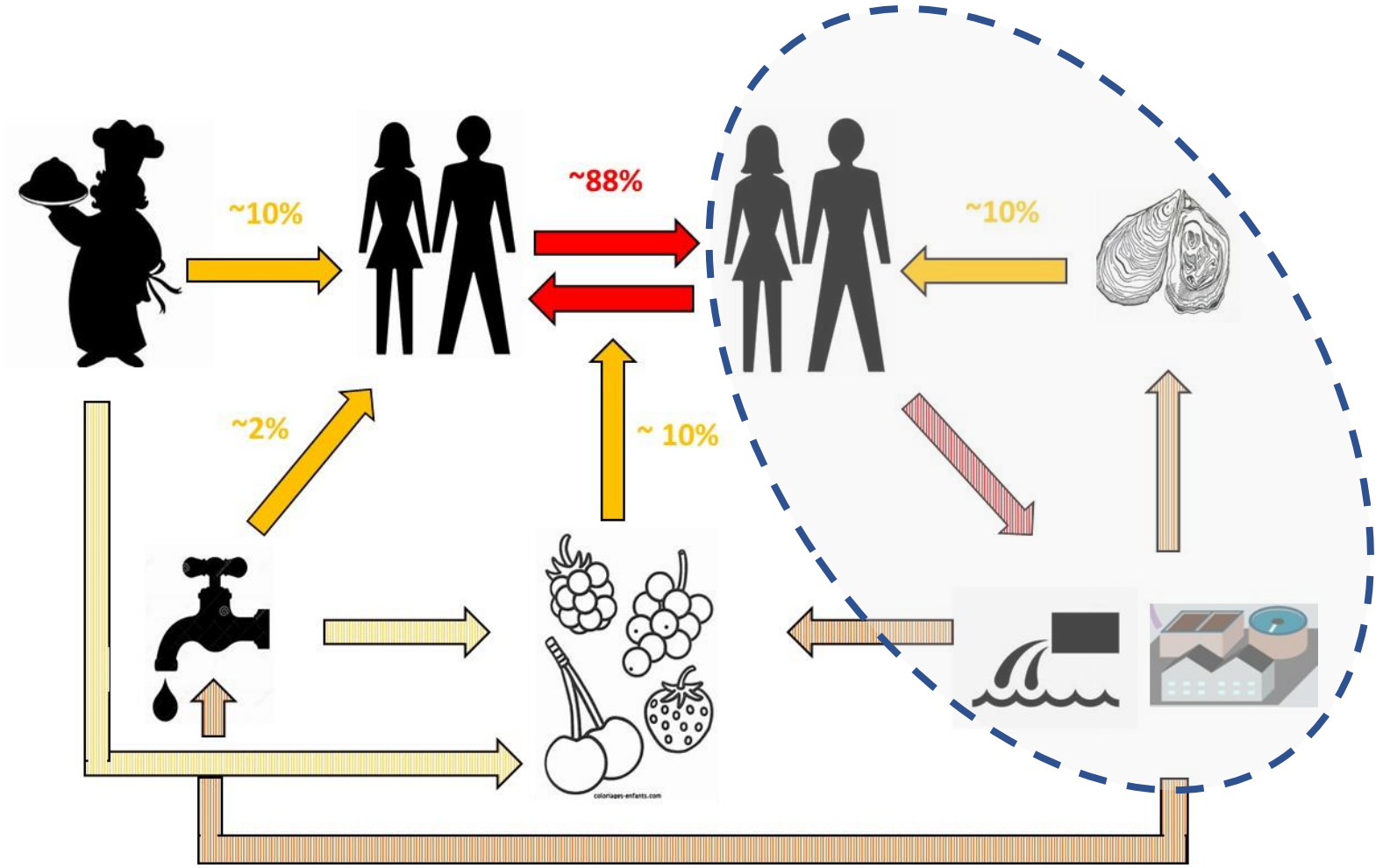
Figure 1. Electron microscopy images of Noroviruses. (**A**). Original image of small round structured viruses, visualized by Kapikian in stool samples from acute gastroenteritis cases, by immune electron microcopy [7]. (**B**). High resolution cryo-electron microscopy of a GII.4 Norovirus particle [9].



Phylogenetic classification of NoV

(Atmar et al., 2008 et 2014; Chhabra et al., 2019; Ettayebi et al., 2021; Kroneman et al., 2008; Lucero et al., 2021)

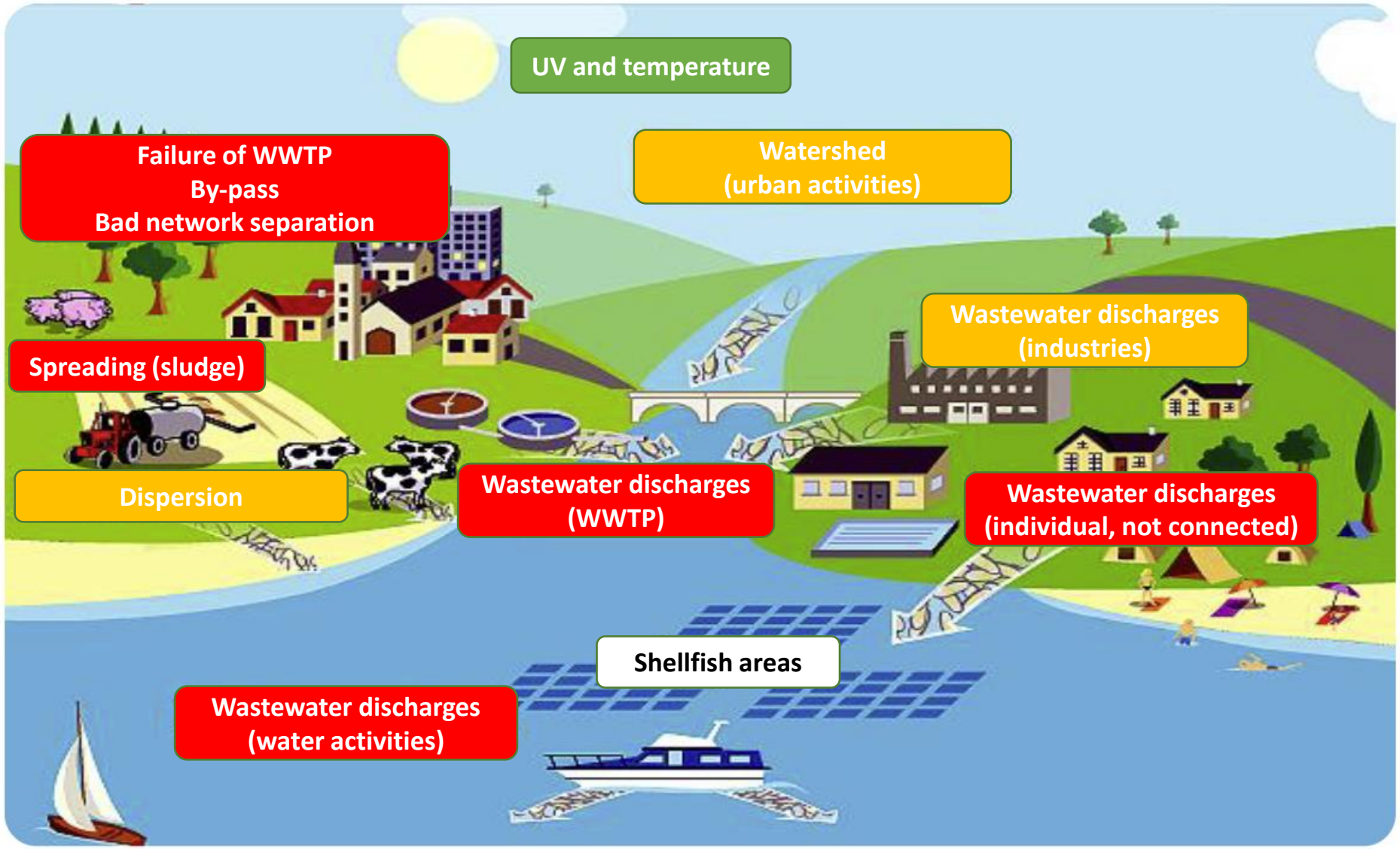
Transmission cycle of NoV



Fecal-oral transmission (direct or indirect) of NoV to humans

(Inspired by Kroneman et al., 2008)

Contamination and inactivation pathways of NoV in coastal areas



(adapted from IFREMER)

NoV foodborne outbreaks in Europe

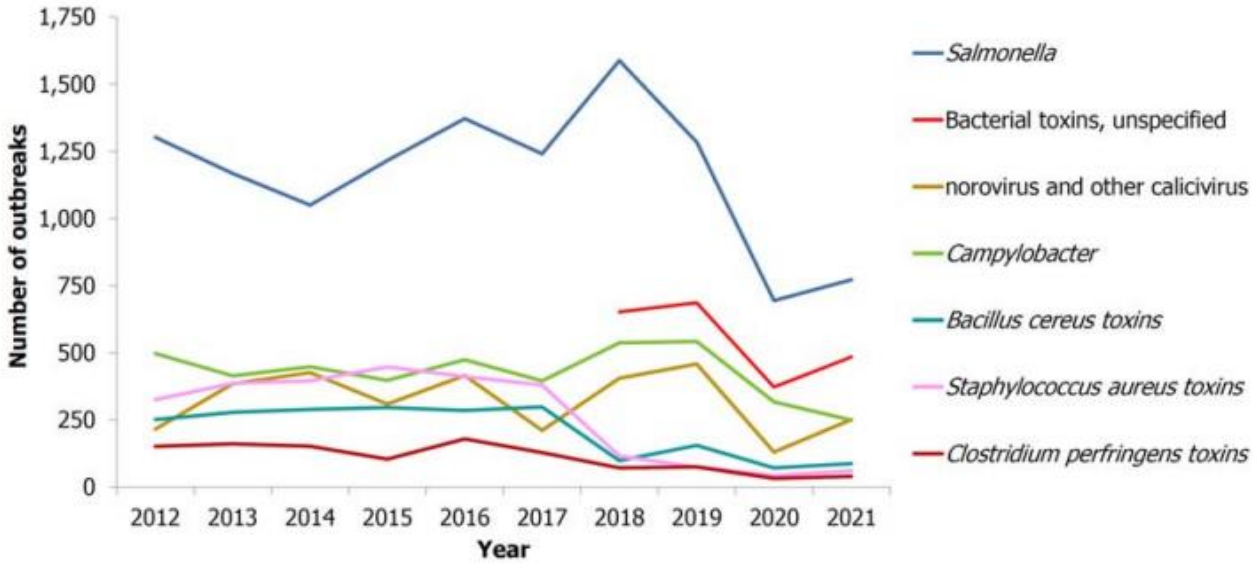


Table 67: Top 10 pathogen/food vehicle pairs causing the highest number of strong-evidence outbreaks in reporting EU MS, 2020

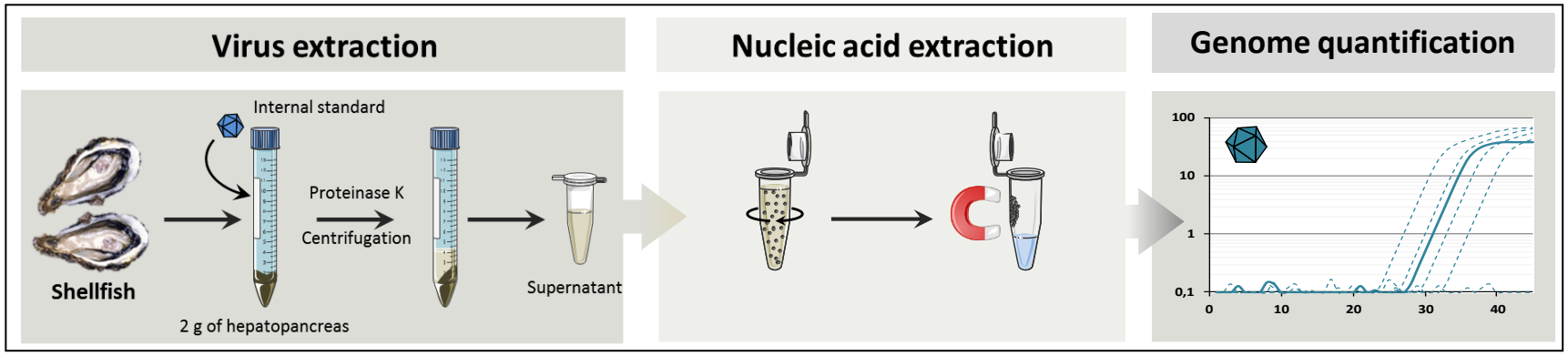
2020					2010–2019[b]		
Rank[a]	Causative agent	Food vehicle	Outbreaks (N)	Reporting MS (N outbreaks)	Rank[a]	Outbreaks (N/year) (range)	Reporting MS (N/year)
1	*Salmonella*	Eggs and egg products[d]	**37**	France (15), Poland (15), Spain (5) Italy (1), Slovakia (1)	1	104.5 (77–141)	10.0
2	Norovirus and other calicivirus	Crustaceans, shellfish, molluscs and products thereof[e]	**28**	France (16), Sweden (9), Denmark (2), Spain (1)	2	36.2 (8–144)	6.1
3	Histamine/ scombrotoxin	Fish and fish products	**14**	Sweden (7), France (4), Germany (2), Belgium (1)	3	31.5 (14–55)	6.9
4	*Salmonella*	Pig meat and products thereof[f]	**11**	France (4), Italy (3), Poland (1), Hungary (1), Belgium (1), Croatia (1)	6	18.9 (9–28)	7.3
5	*Salmonella*	Bakery products[g]	**9**	Poland (9)	4	25.7 (5–45)	4.5
6	*Clostridium perfringens* toxins	Mixed food [h]	**8**	France (2), Denmark (2), Finland (1), Germany (1), Italy (1), Portugal (1)	13	10.3 (3–23)	4.5
7	*Bacillus cereus* toxins	Mixed food[i]	**6**	France (2), Belgium (1), Germany (1), Poland (1), Spain (1)	12	10.9 (7–16)	4.7
7	*Listeria monocytogenes*	Fish and fish products[j]	**6**	Netherlands (2), Denmark (2), Germany (1), Austria (1)	135	0.5 (0–2)	0.4
8	Flavivirus including Tick-borne encephalitis virus	Milk[k]	**5**	Slovakia (4), Austria (1)	65	1.7 (0–3)	1.2
8	Norovirus and other calicivirus	Mixed food[l]	**5**	Denmark (3), Finland (2)	7	14.6 (3–9)	6.3

- **~150 to 500 NoV foodborne outbreaks** each year since 2012 (~ 4% to 20% of the total outbreaks)
- Frequency of the foodborne outbreaks: **2nd position** of the “NoV + BMS” couple

(EFSA, 2022)

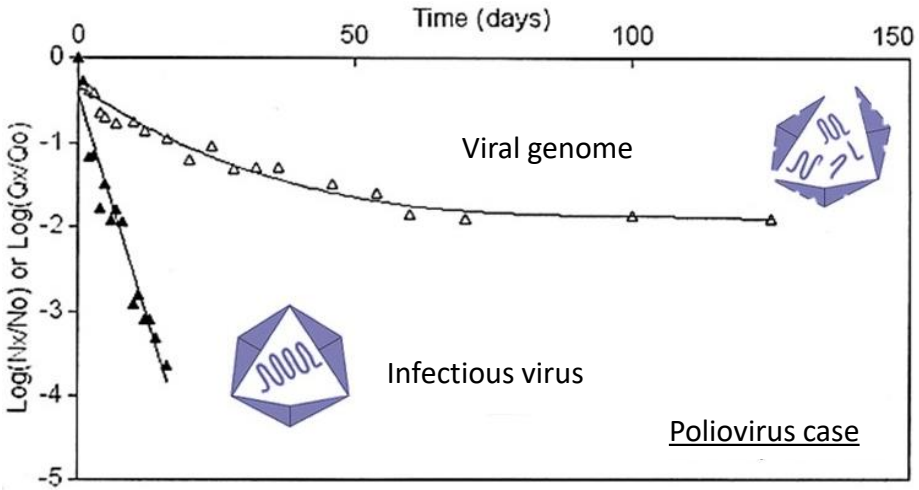
Normative context: advantages and limits

- **ISO 15216 standard** : detection/quantification of NoV genome in BMS
- **NoV prevalence : 34.5% in production areas** (n= 2,180) ; **10.8% in dispatch centres** (n= 2,129)



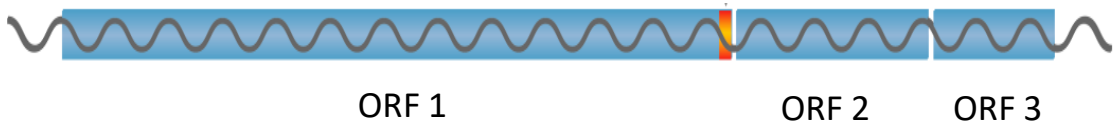
LODt = 80 gc ← 20 gc ← 20 gc ← **1 gc**

Limit 1 : **underestimation** of NoV hazard



Limit 2 : **overestimation** of NoV hazard

region targeted by PCR: ~ 1% of whole genome

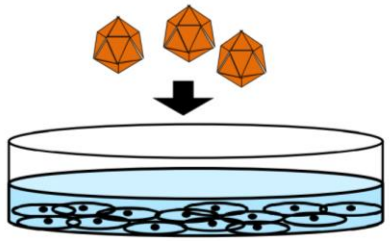


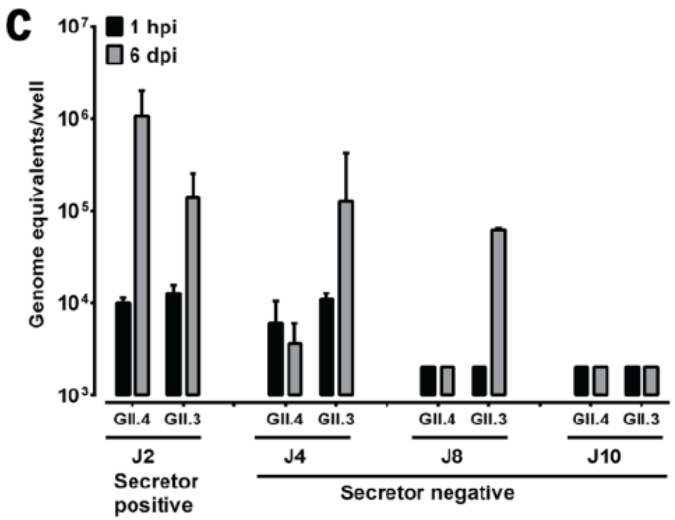
(Gassilloud et Gantzer, 2003; Hartard, 2017; ISO 15216-1 et -2, 2017 et 2019; EFSA, 2019)

Current approaches to detect infectious NoV

2016

In vitro replication (enteroids)





Complex methods

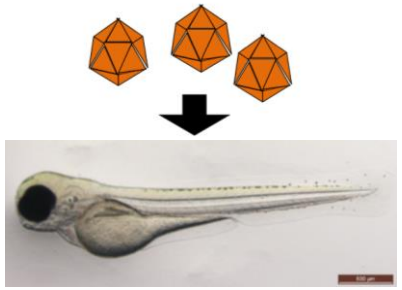
Low sensitivity and specificity

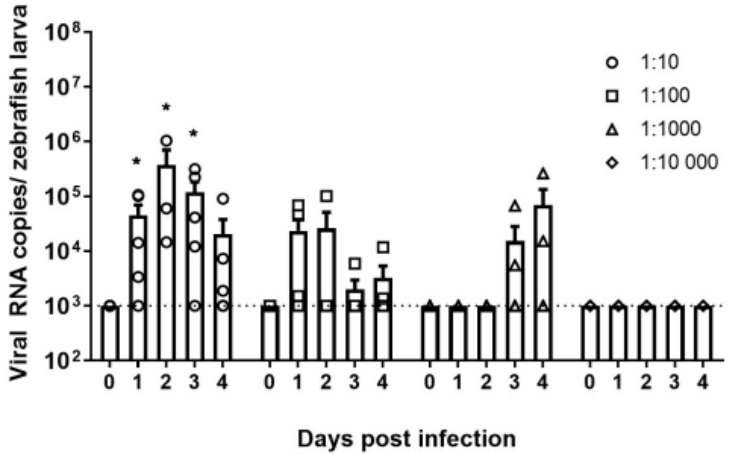
Qualitative result

Not adapted to foodstuff for routine analyses

2019

In vivo replication (*Danio rerio*)

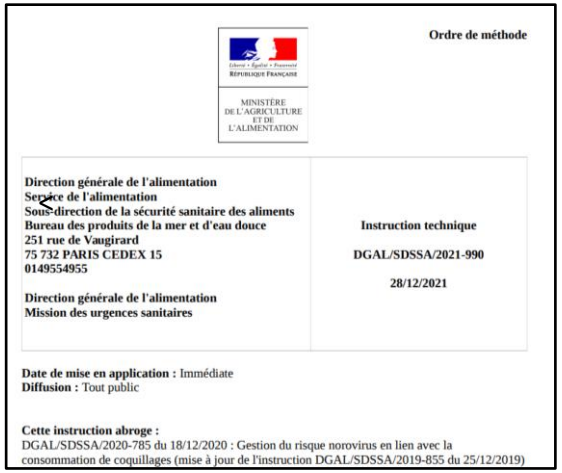




(Ettayebi et al., 2016 et 2019; Van Dycke et al., 2019 et 2021)

Regulatory context in Europe

- **UE 2015/2285** and **CE 854/2004 Regulations**: shellfish areas ranking
- **No regulatory criteria in Europe** for NoV hazard in shellfish
- **French Technical Instruction for NoV in shellfish** since 2013
  - *If NoV foodborne outbreak + NoV contamination of shellfish area => closure +/- 28 days*
- European discussion: evolution of the **853/2004 Regulation**
  - *Integration of NoV in health control plans*
  - *Sanitary studies in shellfish areas / surveillance*



Ordre de méthode

MINISTÈRE DE L'AGRICULTURE ET DE L'ALIMENTATION

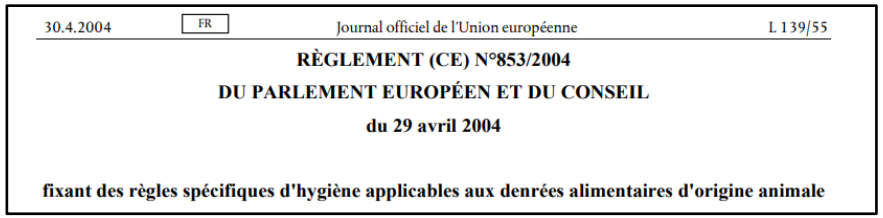
Direction générale de l'alimentation
Service de l'alimentation
Sous-direction de la sécurité sanitaire des aliments
Bureau des produits de la mer et d'eau douce
251 rue de Vaugirard
75 732 PARIS CEDEX 15
0149554955

Direction générale de l'alimentation
Mission des urgences sanitaires

Instruction technique
DGAL/SDSSA/2021-990
28/12/2021

Date de mise en application : Immédiate
Diffusion : Tout public

Cette instruction abroge :
DGAL/SDSSA/2020-785 du 18/12/2020 : Gestion du risque norovirus en lien avec la consommation de coquillages (mise à jour de l'instruction DGAL/SDSSA/2019-855 du 25/12/2019)



30.4.2004 FR Journal officiel de l'Union européenne L 139/55

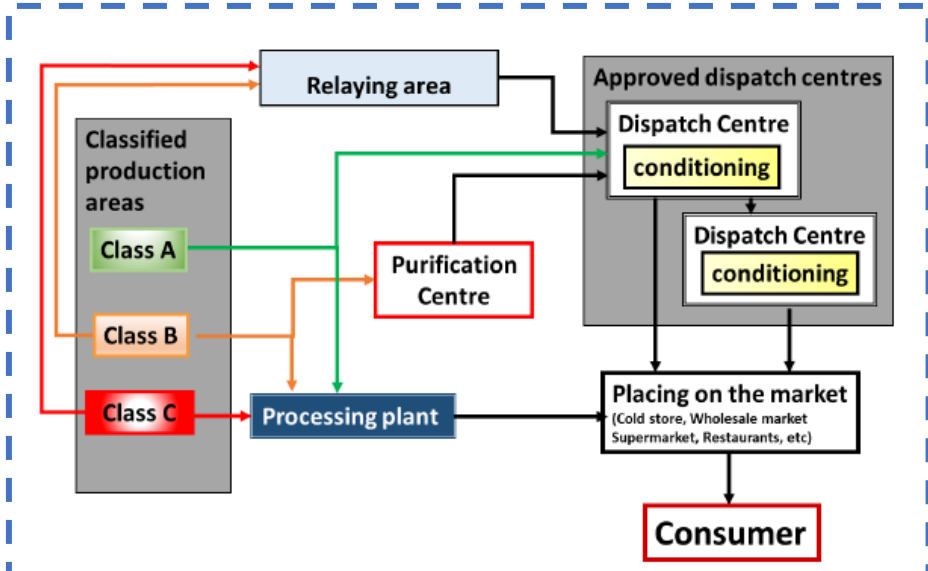
RÈGLEMENT (CE) N°853/2004
DU PARLEMENT EUROPÉEN ET DU CONSEIL
du 29 avril 2004

fixant des règles spécifiques d'hygiène applicables aux denrées alimentaires d'origine animale

L 139/206 FR Journal officiel de l'Union européenne 30.4.2004

RÈGLEMENT (CE) N° 854/2004 DU PARLEMENT EUROPÉEN ET DU CONSEIL
du 29 avril 2004

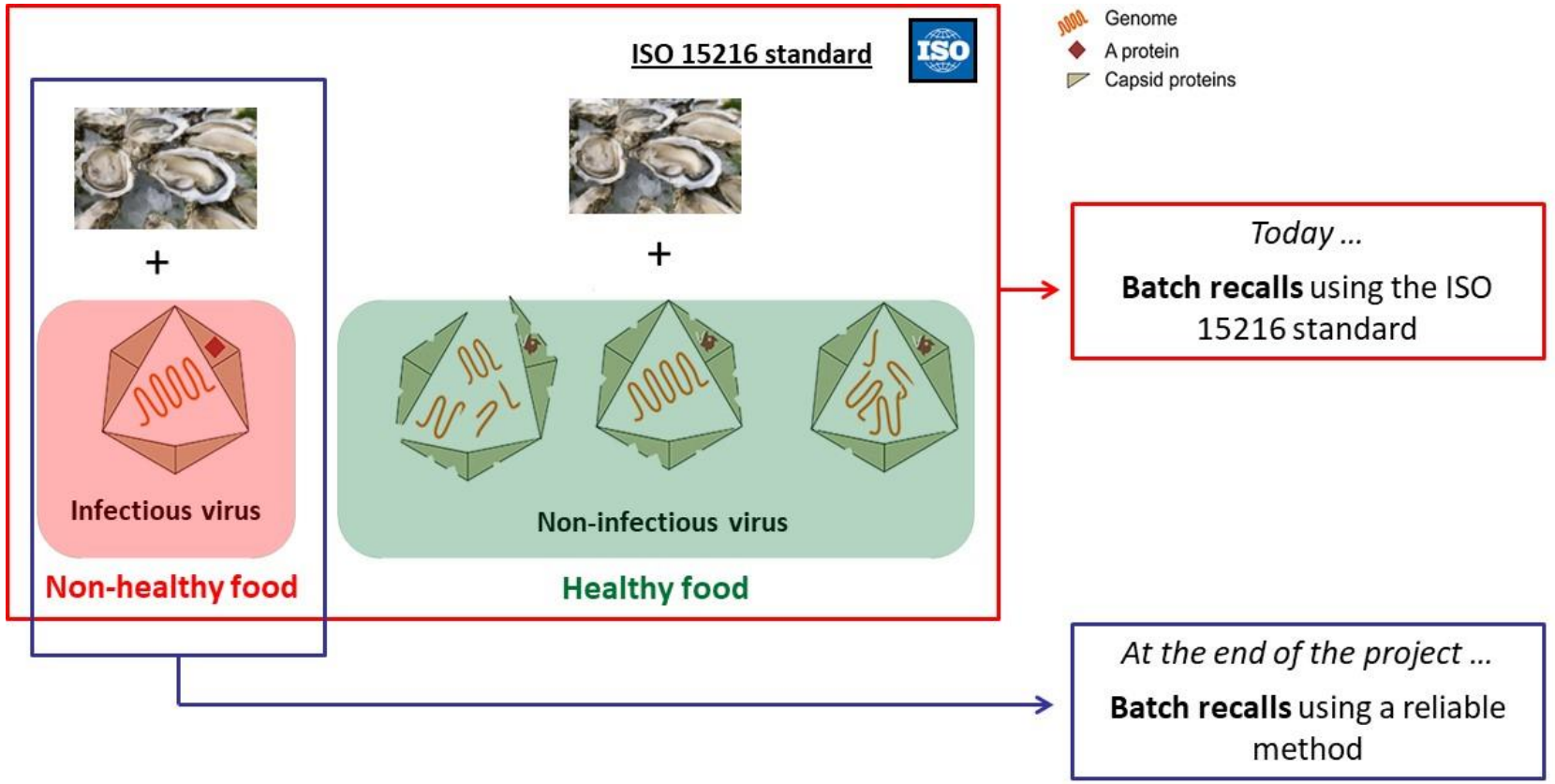
fixant les règles spécifiques d'organisation des contrôles officiels concernant les produits d'origine animale destinés à la consommation humaine



	E.coli / 100 g FIF	Number of areas
Class A	80% < 230 None > 700	103
Class B	90% < 4,600 None > 46,000	248
Class C	100% < 46,000	10
Unclassified	> 46,000	-

(European commission, 2015; DGAL, 2022; ESFA, 2016; European Parliament, 2004)

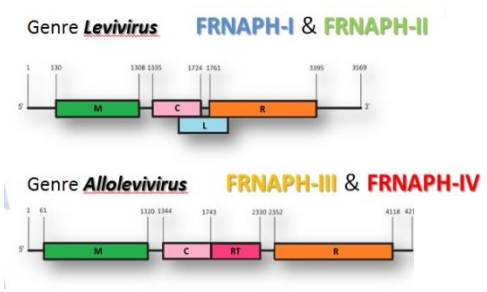
OXYVIR 2 project: objective

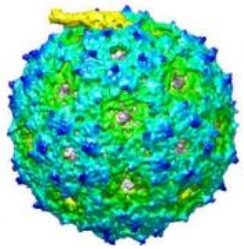


OXYVIR

(Boudaud and Gantzer, 2015)

Discrimination of infectious from non-infectious NoV: evaluation and validation of a viral indicator





Structure of F-Specific RNA bacteriophages

Structure of NoV

~ 3.5 to 4.2 kb	***Single-stranded positive sense RNA***	~ 7.5 kb
~ 30 nm	***Naked virus***	~ 40 nm
90 dimers + A2 protein	***Capsid of icosahedral symmetry***	90 dimers of VP1 + few copies of VP2

- Like *E. coli*, application of the **Mossel concept** (1983) to validate the OXYVIR indicator:
  1. *"must always be detected when the pathogen is detected"*
  2. *"can be detected when the pathogen is not detected"*

Marker (index and indicator) organisms in food and drinking water. Semantics, ecology, taxonomy and enumeration

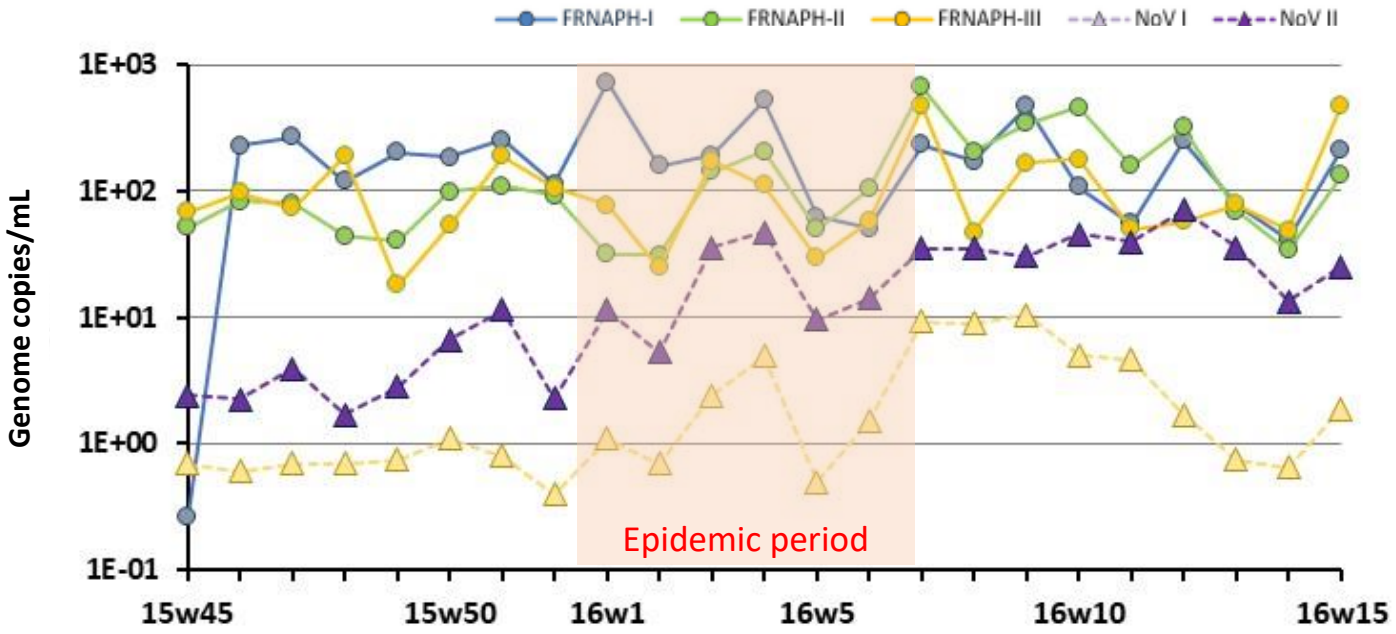
D. A. A. Mossel

(Dent et al., 2013; Donaldson et al., 2010; King et al., 2012; Koning et al., 2016; Lucero et al., 2021; Mossel, 1983)

Features of F-specific RNA bacteriophages (FRNAPH)

- **Indicator of viral pollution:**
  - Abundant in raw urban wastewater (~10^{6-7} PFU/L), despite a low prevalence in human stools (< 26%)
  - Survival in the environment: FRNAPH-I > **FRNAPH-II** >> FRNAPH-III > FRNAPH-IV
  - Human origin of **FRNAPH-II** and –III
  - Included in the **Australian** and **US regulations** to control the microbiological quality of BMS (threshold at 50 PFU/100 g FIF)
  - **FRNAPH-II genomes** (10^{7-8} copies/L) **> NoV genomes** (10^{4-7} copies/L) in raw urban wastewater

Treated wastewater



(ASQAAC, 2016; Calci et al., 1998; FDA, 2015; Fitzmorris-Brisolara et al., 2022; Grabow et al., 1995; Hartard et al., 2015; Hartard, 2017; Havelaar et al., 1990; Lee et al., 2018; Schaper et al., 2002)

Validation of the OXYVIR indicator: comparison of FRNAPH-II genomes and NoV genomes in oysters

In the framework of **prevalence studies**

TABLE 3 Contingency table for detection of FRNAPH-II and NoV GII genomes in oyster batches

FRNAPH-II genome detection	No. of samples with the following result for NoV GII genome detection:		
	−	+	++
−	49	9	1
+	11	13	7
++	5	7	9

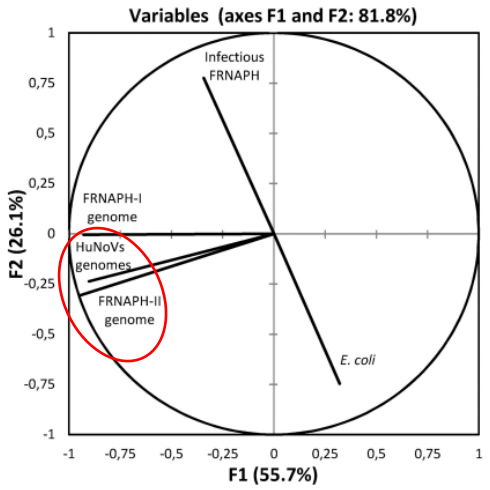
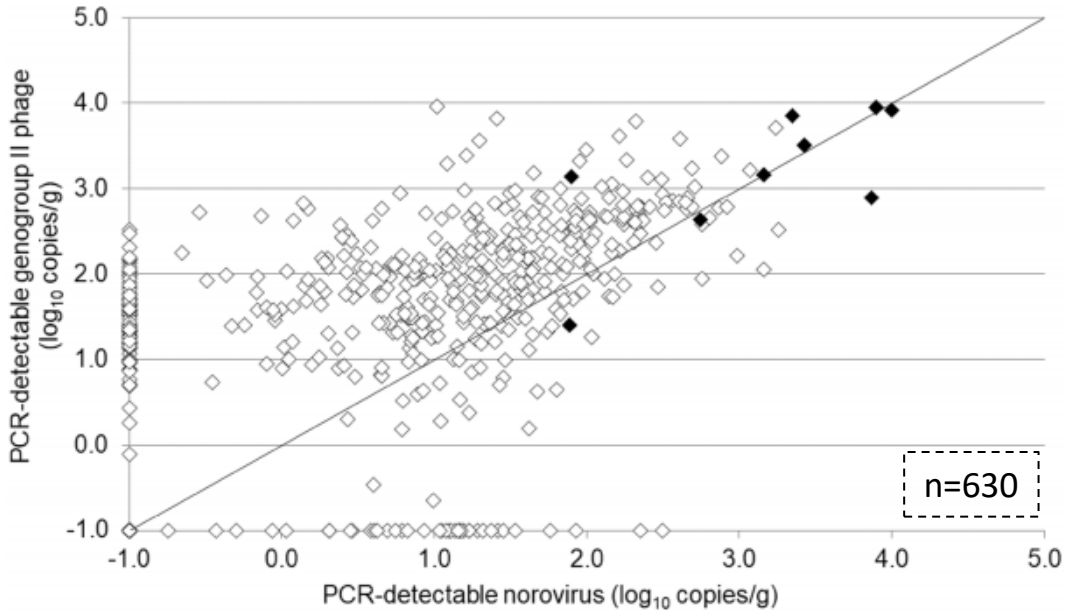


FIG 4 Principal-component analysis of parameters targeted in shellfish collected from the local harvesting area.

n=111



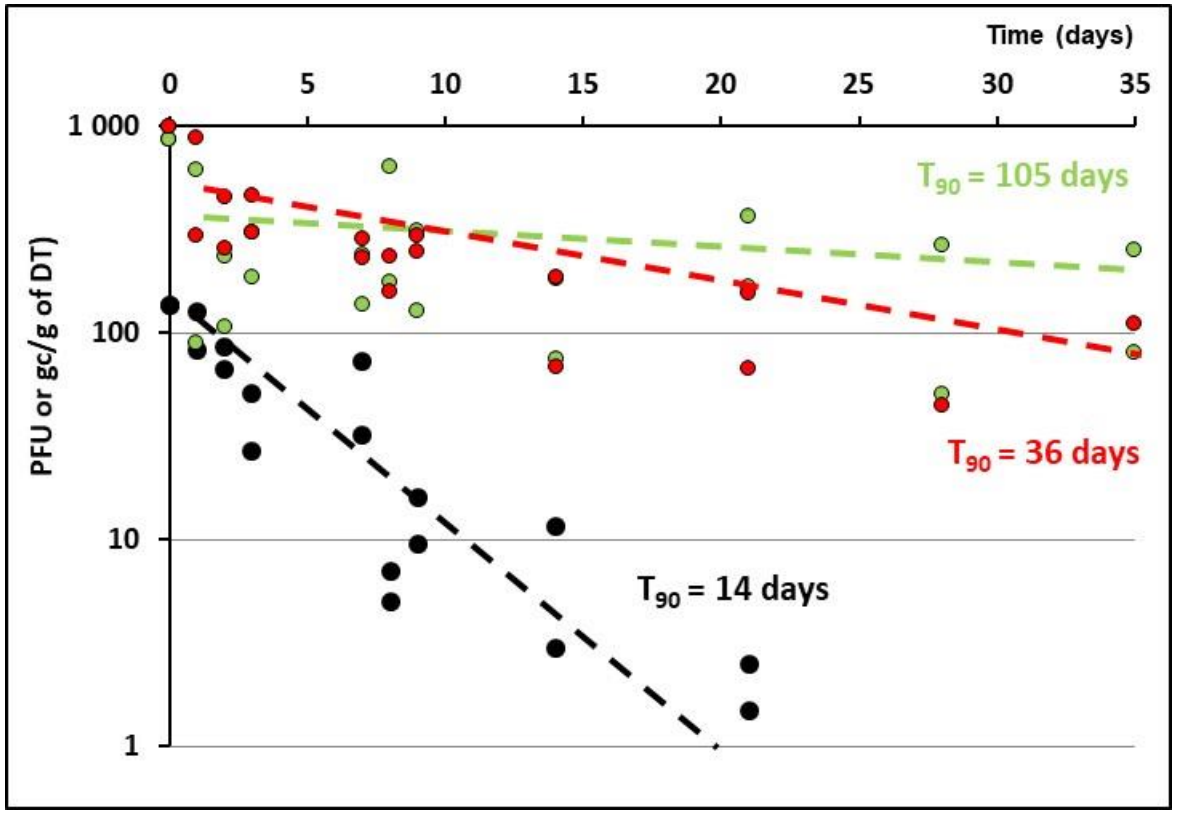
- **Significant correlation** between FRNAPH-II and NoV GII (p-value < 10^{-4})

- Confirmed by the CEFAS (ex-EURL “NoV – shellfish”) and others studies

(Hartard et al., 2016 et 2018; Lowther et al., 2019; Gyawali et al., 2021)

Validation of the OXYVIR indicator: comparison of NoV (genome) and FRNAPH (infectious and genome) in oysters

In the framework of **oyster depuration at industrial scale**

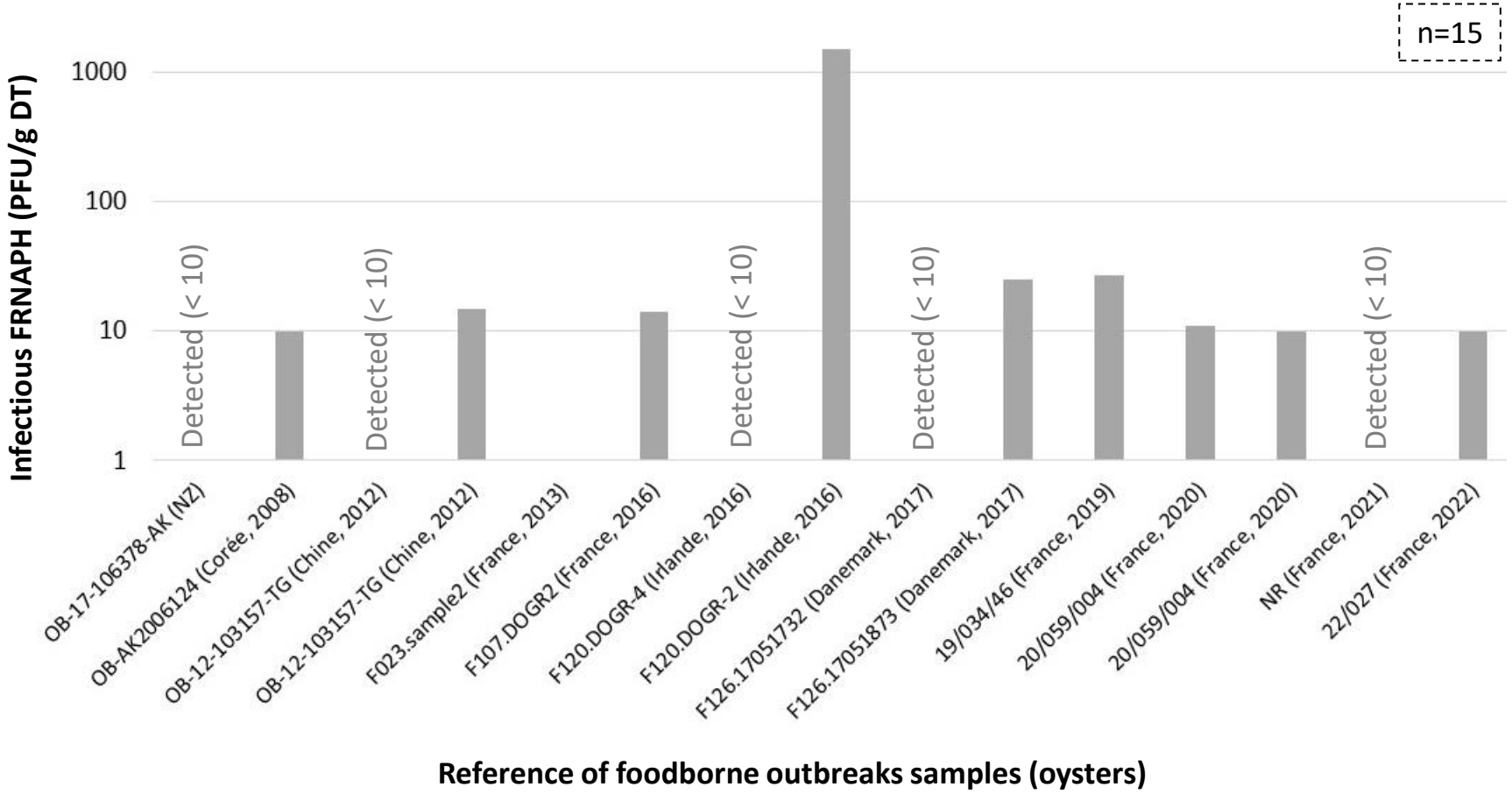


n=6

- The detection of viral genome cannot always predict the presence of infectious particles for a specific virus
- Results confirmed by other published studies

(Hartard et al., 2018; Leduc et al., 2020; Younger et al., 2020; Gyawali et al., 2021)

Validation of the OXYVIR indicator: comparison of infectious FRNAPH and infectious NoV in oysters (foodborne outbreaks)

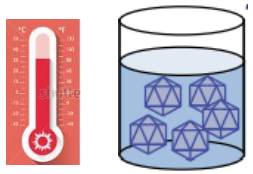


- Detection of infectious FRNAPH in **100%** of oysters responsible of acute gastroenteritis caused by NoV

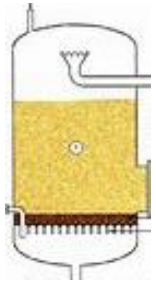
(Boudaud et al., in preparation)

Validation of the OXYVIR indicator: comparison of infectious FRNAPH and infectious NoV at a laboratory-scale

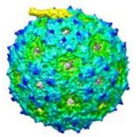
10 to 20°C in PBS solution at 150 mM (120 days)



Water at 20 and 35 g/L NaCl at 20°C (120 days)



NoV



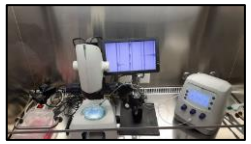
FRNAPH-II

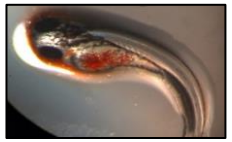
Infectivity ?

Sand filtration at 56 m/h (water at 35 g/L NaCl)

UV_{254nm} fluence (water at 35 g/L NaCl): 0 to 60 mJ/cm²

Validation of the indicator if survival of infectious FRNAPH-II ≥ infectious NoV



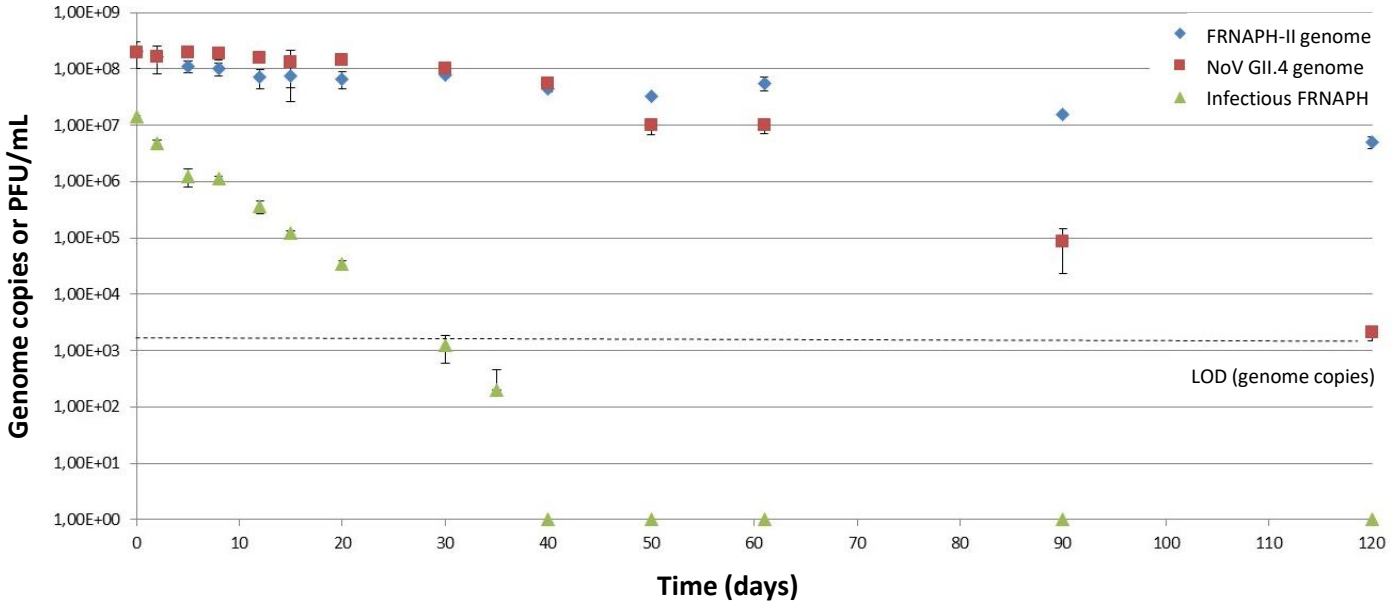



norme européenne
NF EN ISO 10705-1
Octobre 2001
norme française
Qualité de l'eau
Détection et dénombrement des bactériophages

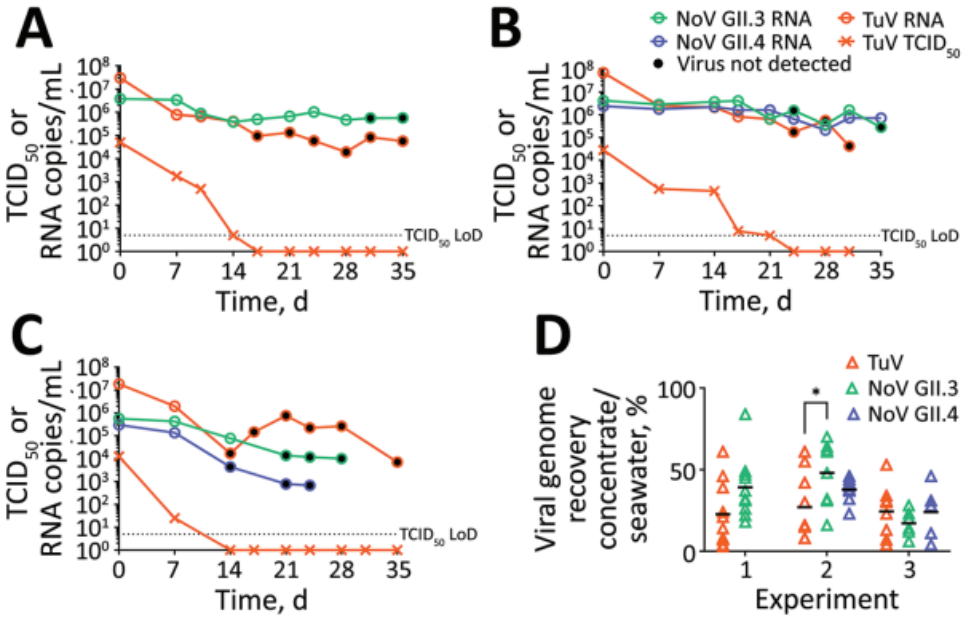
(ISO 10705-1, 2001 ; Van Dycke et al., 2019 et 2021 ; Wolf et al., 2010)

Survival of infectious FRNAPH and infectious NoV at a laboratory-scale

20°C; PBS solution at 150 mM



12°C; seawater (3 conditions)



- Similar inactivation of infectious FRNAPH-II and infectious NoV?

(Robin et al., in preparation ; Desdouits et al., 2022)

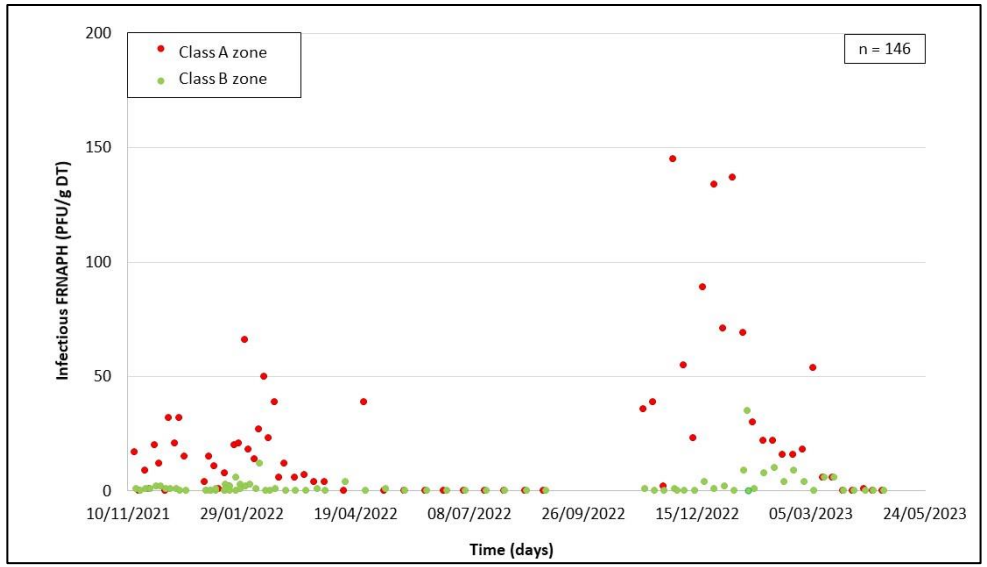
Validation of the OXYVIR indicator in the field

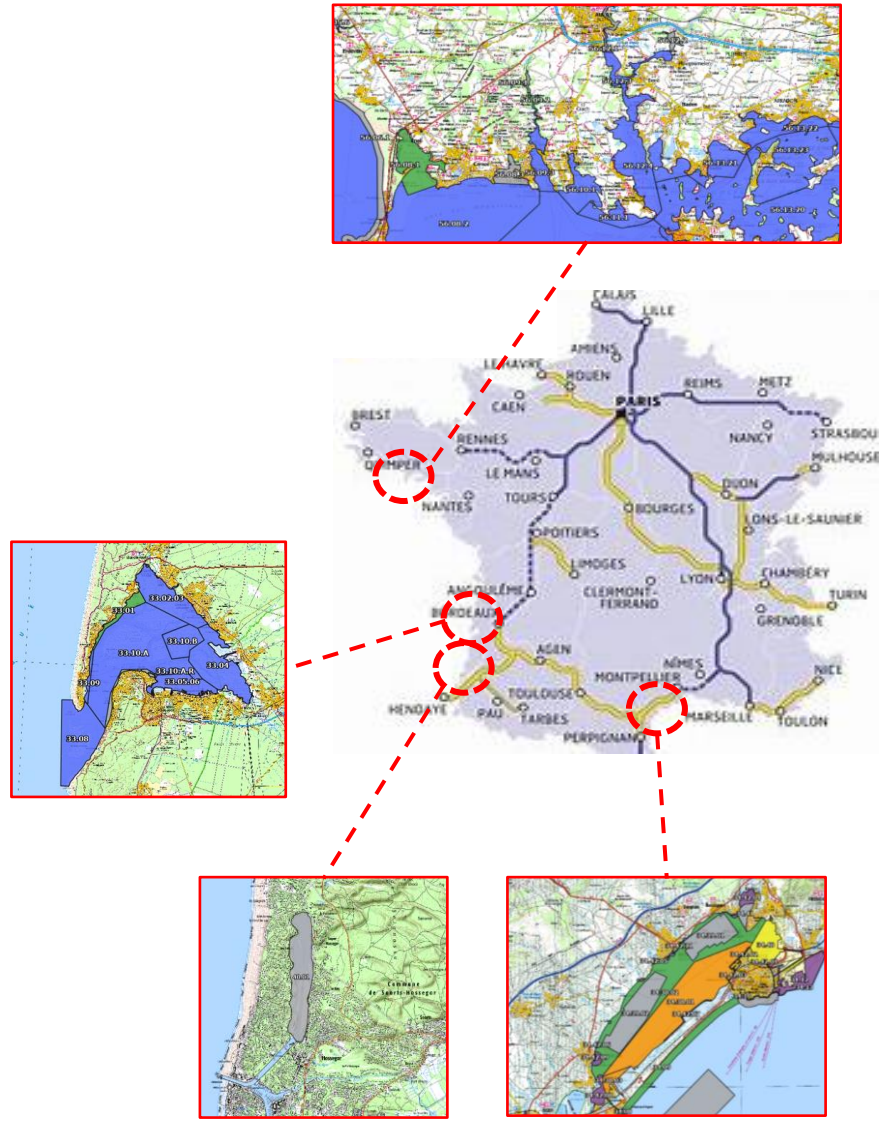
Strengthening correlation between infectious FRNAPH and infectious NoV in oysters

Determinating infectious FRNAPH threshold in oysters

Anticipating anomalies related to the contribution of viral pollution in oysters

Validation of a NoV management protocol





in progress…

COMITÉ NATIONAL DE LA CONCHYLICULTURE

Validation of the OXYVIR indicator in the field

Specific / non-specific interactions between viral particles and digestive tissues of oysters

	NoV	FRNAPH-II
Specific	A-like HBGAs, sialic acids	None
Non-specific	Charge, hydrophobicity	

Prevalence of infectious FRNAPH in human population

	FRNAPH (infectious and genome)
Clinical study PRI-3 (100 fresh stools)	Proportions and contents

Behavior of viral particles in oysters

CHU ROUEN NORMANDIE

Soumission d'un projet de Recherche

Vous souhaitez mener une recherche. Merci de compléter ce document et l'envoyer à l'adresse suivante : secretariat.DRC@chu-rouen.fr

Porteur du projet	
Nom / Prénom	Favennec Loic
Etablissement :	CHU de Rouen
Service	Parasitologie mycologie
Téléphone :	0232881455
Mail :	loic.favennec@chu-rouen.fr

Titre (même provisoire)

Evaluation de la prévalence du portage de phages fécaux (bactériophages ARN F-spécifiques et coliphages somatiques) dans les selles de patients sans pathologie infectieuse

(Le Guyader et al., 2006 ; Maalouf et al., 2010)

Conclusions

- **Enteric origin** and **similar structures** of FRNAPH and NoV
- FRNAPH-II genome > NoV genome in **wastewaters** (raw and treated)
- **Similar and low decays** of FRNAPH and NoV genomes in the **environment** and during the **oyster depuration**
- **Correlation** well demonstrated between FRNAPH-II genome and NoV genome in **oysters**
- **Similar survival** of infectious FRNAPH and NoV in oysters partially demonstrated:
    1. Results of 15 oyster samples responsible of acute gastroenteritis caused by NoV
    2. Infectious FRNAPH criteria used in routine by many French oysters farmers (no negative customer returns)
    3. Literature data
    4. Laboratory studies in progress in the framework of the OXYVIR project

Outlooks:
control of NoV hazard in oysters through using the OXYVIR indicator