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FOOD SAFETY IMPLICATIONS FROM THE USE OF ENVIRONMENTAL INHIBITORS IN AGRIFOOD SYSTEMS

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FOREWORD

Climate crisis and increasing food demand accelerate the need for agrifood systems to transform to meet the 2030 Agenda for Sustainable Development. Numerous approaches are being explored to reconcile the intensification of agrifood production with mitigation measures aimed at lowering negative impacts on the environment and ensuring sustainability. Trade-offs between environmental, socioeconomic, food security and safety objectives are a challenge of today's sustainable development and resilience of agrifood systems to shocks and stresses.

In this context, environmental inhibitors are among the options that are currently being explored and used. Environmental inhibitors are substances that are used to reduce greenhouse gas emission from the livestock sector or to minimize the loss of nutrients from cultivated soil which has additional benefits in improved performance of animals and efficiency of fertilizers. While they are not new to agricultural practices, their implementation needs to consider many aspects starting from possible food safety concerns flanked by the limited information available in this regard, as well as their implications for trade and economies.

Developing integrated strategies to create synergies between involved sectors is at the basis of policies targeting smart agriculture in agrifood systems under transformation. The Strategic Framework of the Food and Agriculture Organization (FAO) of the United Nations, centered around better production, better nutrition, a better environment, and a better life, supports the 2030 Agenda, where safe food for everyone is an important priority area. Scientific advancement and innovation are key in providing means, either in terms of knowledge or technological improvements, to fill current gaps which impede an optimal risk assessment and implementation of new solutions for a more sustainable production of safe food.

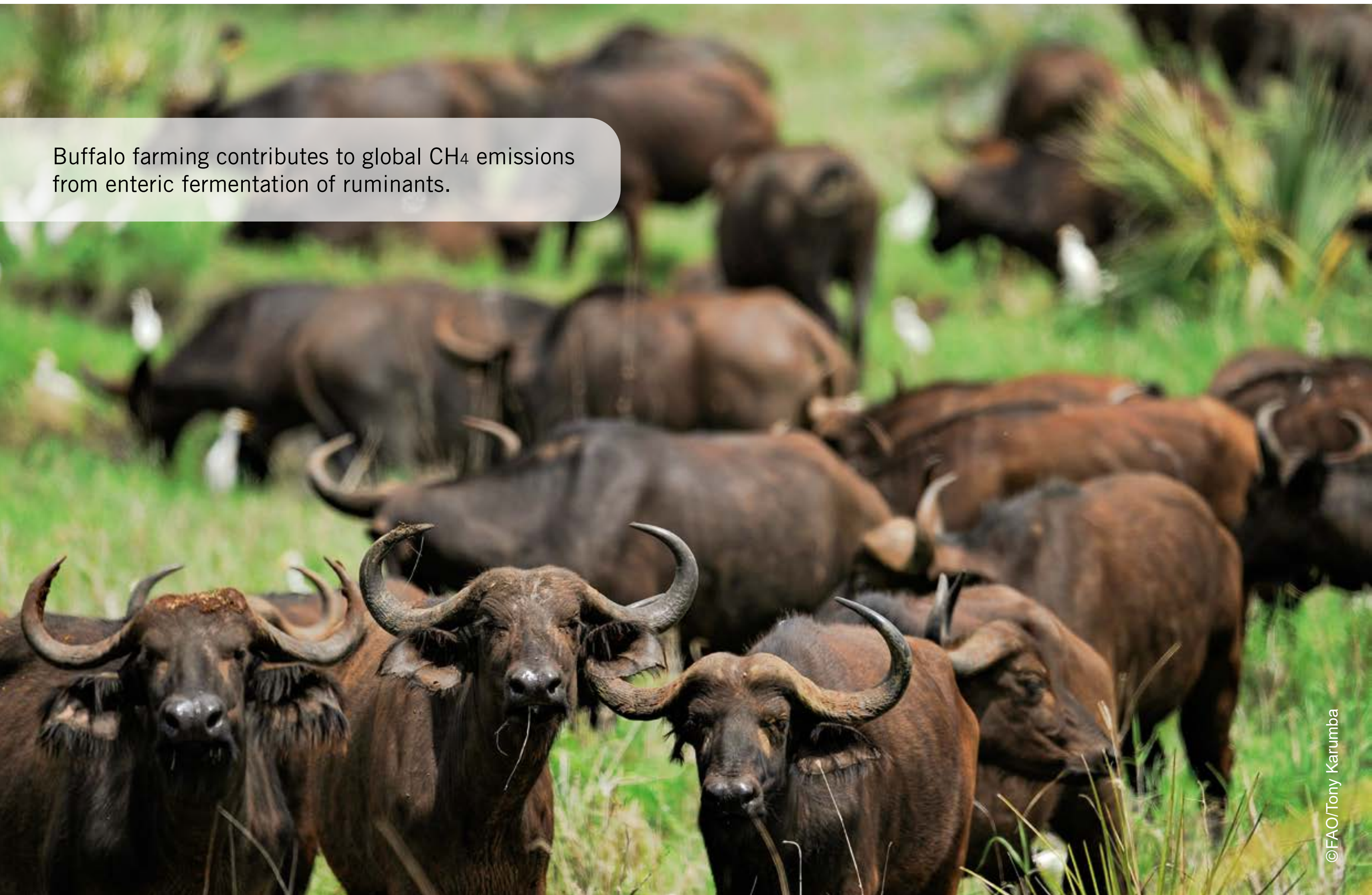
This report provides a food safety analysis of the main groups of environmental inhibitors. It also offers an overview of how different regulatory frameworks worldwide deal with environmental inhibitors, highlighting at the same time the lack of global harmonization in defining and categorizing these substances.

We hope that the findings and conclusions of this report will offer some viable options and recommendations to move forward in realizing the full potential of environmental inhibitors towards resilient, sustainable and safe agrifood systems.

Corinna Hawkes
Director
Food Systems and Food Safety Division

Flooded rice fields are a particular type of cultivation where the need for fertilizers and anaerobic environment lead to higher CH_4 emissions compared to other crops.

©FAO/Mohammad Rakibul Hasan



Buffalo farming contributes to global CH_4 emissions from enteric fermentation of ruminants.

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The document was technically edited by Rosa Abruzzese, while graphic design was provided by Tomaso Lezzi.

ABBREVIATIONS

ACVM	Agricultural Compounds and Veterinary Medicines Act 1997 of New Zealand
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
AMR	antimicrobial resistance
AOA	ammonia oxidizing archaea
AOB	ammonia oxidizing bacteria
ARfD	acute reference dose
ATSDR	Agency for Toxic Substances and Disease Registry
BDI	biological denitrification inhibitors
$BMDL_{10}$	benchmark dose lower bound with 10 percent additional risk of adverse effects
BNI	biological nitrification inhibitors
CCPR	Codex Committee on Pesticide Residues
CCRVDF	Codex Committee on Residues of Veterinary Drugs in Foods
CDC	Centers for Disease Control and Prevention
CO_2-eq	carbon dioxide equivalent
Comammox	COMplete AMMonia OXidation
ECHA	European Chemicals Agency
EDI	estimated daily intake
EEA	European Economic Area
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FARM	Argentine Federation of Rural Associations of Mercosur
FAS	Scotland’s Farm Advisory Service
FDA	United States Food and Drug Administration
FEPALE	Pan-American Dairy Federation
FSAI	Food Safety Authority of Ireland
GHG	greenhouse gas
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
GRAS	generally recognized as safe
GWP	global warming potential
HAB	harmful algal bloom
HMA	Halogenated methane analogues

IAEA	International Atomic Energy Agency
IARC	International Agency for Research on Cancer
IEDI	internationally estimated daily intake
IFA	International Fertilizer Association
ILO	International Labour Organization
IOC	Intergovernmental Oceanographic Commission – UNESCO
IPCC	Intergovernmental Panel on Climate Change
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
LOQ	limit of qualification
MARA	Ministry of Agriculture and Rural Affairs of China
MCFA	medium-chain fatty acids
MRL	Maximum Residue Limit
MSDI	maximized Survey-derived Daily Intake
NICNAS	Australian National Industrial Chemicals Notification and Assessment Scheme
NMPF	The National Milk Producers Federation
NOB	Nitrite oxidizing bacteria
NUE	nitrogen use efficiency
OECD	Organisation for Economic Co-operation and Development
PFC	Product Function Category under Regulation (EU) 2019/1009
PGgRc	Pastoral Greenhouse Gas Research Consortium of New Zealand
POPs	Persistent Organic Pollutants
PUFA	Polyunsaturated fatty acids
RfD	reference dose
SDG	Sustainable Development Goals
SENASA	National Food Safety and Quality Service of Argentina
SRA	Argentine Rural Society
TDI	tolerable daily intake
TDM	triazole derivative metabolite
UNECE	United Nations Economic Commission for Europe
UNEP	United Nations Environment Programme
UN	United Nations
USDEC	Council of Dairy Exporters of United States
USEPA	United States Environmental Protection Agency
VFA	volatile fatty acids
VKM	Norwegian Scientific Committee for Food Safety
VMD	Veterinary Medicine Directorate of the UK
WHO	World Health Organization

Chemical formulae

2,4,6-TCP	2,4,6-trichlorophenol
2,4-DPC	2,4-dichlorophenol
3-NOP	3-nitrooxypropanol
6-CPA	6-chloropicolinic acid
AITC	allyl isothiocyanate
AMO	ammonia monooxygenase
BCM	bromochloromethane
BDCM	bromodichloromethane
BES	2-bromoethanesulfonate
BPS	3-Bromopropanesulfonate
C-1	(4-hydroxyphenyl) chloromethanesulfonate
CES	2-Chloroethanesulfonate
CH_3COOH	acetic acid
CH_4	methane
ChE	cholinesterase
CMP	1-carbamoyle-3-methylpyrazole
$CO(NH_2)_2$	urea
CO_2	carbon dioxide
DBCM	dibromochloromethane
DCD	dicyandiamide
DCS	N-2,5-dichlorophenyl succinamic acid
DMP	3,4-dimethylpyrazole
DMPP	3,4-dimethylpyrazole-phosphate
DMPSA	3,4-dimethylpyrazole succinic acid
EQI	ethoxyquin quinone imine
EQNL	2,4-Dimethyl-6-ethoxyquinoline
H_2	hydrogen
H_2S	hydrogen sulfide
HAO	hydroxylamine oxidoreductase
HCR	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HDMBOA	2-hydroxy-4,7-dimethoxy-2*H*-1,4-benzoxazin-3(4*H*)-one
HEPA	2-hydroxyethane phosphonic acid
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
MCR	methyl-coenzyme M reductase
Me-CoM	methyl-coenzyme M
MES	2-Mercaptoethanesulfonate
MHPP	methyl 3-(4-hydroxyphenyl) propionate
MPA	N-[3(5)-methyl-1*H*-pyrazol-1-yl)methyl]acetamide

MTR	**coenzyme M methyltransferase**
N_2O	**nitrous oxide**
NBPT	**N-(n-butyl) thiophosphoric triamide**
NBPTo	**N-(n-butyl)phosphoric triamide**
NH_3	**ammonia**
NH_4^+	**ammonium**
NirK	**copper nitrite reductase from** *Fusarium oxysporum*
NO_2^-	**nitrites**
NO_3^-	**nitrates**
NO	**nitric oxide**
NOPA	**3-nitrooxypropionic acid**
NOR	**Nitric oxide reductase**
NPPT	**N-(n-propyl) thiophosphoric triamide**
PCP	**pentachlorophenol**

EXECUTIVE SUMMARY

Agrifood systems are under unprecedented pressure to produce more food for the growing world population, while reducing the environmental footprint and coping with the challenges posed by climate change. In this context, numerous practices and technologies are being developed to increase the sustainability of agrifood systems. One of the approaches to reduce negative impacts on the environment while at the same time improving the production efficiency of crops and livestock, is the application of the so called "environmental inhibitors", in particular:

> methanogenesis inhibitors: these reduce methane emissions resulting from enteric fermentation of ruminants and from other agricultural sources (such as rice paddies or manure), and

> nitrogen inhibitors: these limit the loss of nitrogen from farmlands by slowing down natural processes which lead to its leakage and volatilization.

While the use of methanogenesis inhibitors is currently mostly limited to research purposes, several products are available on the market as nitrogen inhibitors. It is likely that the intensification of livestock farming and use of nitrogen fertilizers in the coming years will increase the application of nitrogen inhibitors. High fertilizer prices may also encourage the use of nitrogen inhibitors to minimize fertilizer demand while maintaining crop yield and quality.

This publication aims to provide an overview of substances used as environmental inhibitors and to summarize information on potential implications on food safety and regulations resulting from their application.

In many cases, information on potential residues of environmental inhibitors and/or their metabolites in food commodities is not available, which does not allow for thorough food safety risk assessments to be carried out. At the same time, the way environmental inhibitors are being covered by regulatory frameworks worldwide is diverse. Various legal instruments are applied to regulate these compounds at national and regional level, in particular through regulations on feed additives, food additives and substances used in food production, fertilizers and pesticides. Some environmental inhibitors are covered by legislations related to other primary uses (e.g. industrial use, human medicine) when reduction of methane emissions or the loss of nitrogen is the secondary application. As chemical residues of environmental inhibitors can occasionally be detected in food at low levels for which no regulatory limits exist, Codex Alimentarius adopted international guidelines for rapid risk analysis of such substances and agreed that Maximum Residue Limits (MRLs) for inhibitors could be considered by the Codex Committee on Pesticide Residues (CCPR) for crops and by the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) for animals.

Ensuring food safety of environmental inhibitors requires further efforts to close regulatory and knowledge gaps. Science-based holistic risk assessment and internationally harmonized legislative frameworks are needed to ensure food safety of environmental inhibitors and facilitate their global trade. Concurrently, research and development of novel environmental inhibitors needs to proceed in concert with risk assessments. The approval of new products and technologies, such as genetic recombination of crops increasing the capacity of environmental inhibitors contained within plant tissues, requires increasing efforts to evaluate the implications on food safety. On the other hand, re-purposing old chemistry as environmental inhibitors needs a critical examination of new toxicological studies to understand limitations and changes in the data requirements.

New technological solutions can help in optimizing the application of environmental inhibitors. For instance, improvements in the quantification of greenhouse gas (GHG) released from livestock and farmlands can allow a more precise and simple application of these substances on commercial farms. However, while the development of more advanced detection methods for environmental inhibitors or their metabolites in complex matrices can strengthen food safety management, an increasing analytical sensitivity would require regulatory approaches for dealing with higher number of detects in foods. Furthermore, technological progress in other scientific domains, for example in omics technologies applied to understand changes in rumen microbiome, can help investigate natural processes underlying the efficiency of mitigation strategies on methane formation and livestock performance.

Effective implementation of environmental inhibitors needs to take into account multiple factors related to costs, uncertainties and possible food safety risks of their application for farmers, industries and consumers. Those factors depend on local conditions, therefore careful evaluation on a case-by-case basis is considered the best approach to achieve required outcomes while minimizing the negative impact on other variables in the system. Current estimates indicate that costs of inhibitor application may be outcompeted by savings when considering societal benefits related to the reduction of GHG emission for human and ecosystem health. Financial incentives are among the factors predicted to promote the adoption of GHG mitigation strategies in practice.

As the trend shifts towards more sustainable agrifood systems, environmental inhibitors are among the tools that can offer viable solutions towards adequate nutrition for the growing world population while minimizing the impacts on the environment. At the same time, it is critical to keep raising awareness of the various interconnections that exist in agrifood systems, so that any food safety implications are carefully considered and proactively addressed.

The efficacy of fertilizers can be improved by the application of nitrogen inhibitors which reduce the loss of nitrogen from the rootzone of cultivated plants.

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CHAPTER 1

INTRODUCTION AND BACKGROUND

The UN estimates that the global human population will reach 9.7 billion by 2050 (UN, 2022). Projections for periods ranging from 2010 to 2050 show that food demand is forecasted to grow by up to 60 percent (Falcon, Naylor and Shankar, 2022; van Dijk *et al.*, 2021). At the same time, intensifying food production and the corresponding supply chains can lead to increased greenhouse gas (GHG) emissions which has an enormous impact on climate change (see **Figure 1**) (Mbow *et al.*, 2019; Opio *et al.*, 2013). If no action is taken, GHG emissions from agrifood systems are expected to grow by 30-40 percent over the next three decades (Mbow *et al.*, 2019). International efforts are being made to address global warming and set time checkpoints through seventeen Sustainable Development Goals (SDG)[1] and the Paris Agreement,[2] involving the reduction of GHG emissions by 2030. Moving towards sustainable agrifood systems is high on the agenda of the United Nations as also demonstrated by the recent FAO Global Conference on Sustainable Livestock Transformation.[3]

Against this backdrop, numerous practices and technologies are being developed to increase sustainability of agrifood systems. Environmental inhibitors are one such approach, as they are used to reduce negative impacts on the environment while improving at the same time the production efficiency of crops and livestock. In this publication, the term environmental inhibitors is used to describe anthropogenic or naturally occurring substances (or their mixtures) that are used to reduce:

- GHG emissions, in particular methane (CH_4), from livestock, manure (see **Box 1**) as well as croplands by blocking key enzymes or pathways in methanogenic microorganisms which limits their growth and ability to produce CH_4;
- loss of nitrogen from soil by delaying or blocking the activity of specific enzymes or microorganisms responsible for nitrification, denitrification or urea hydrolysis.

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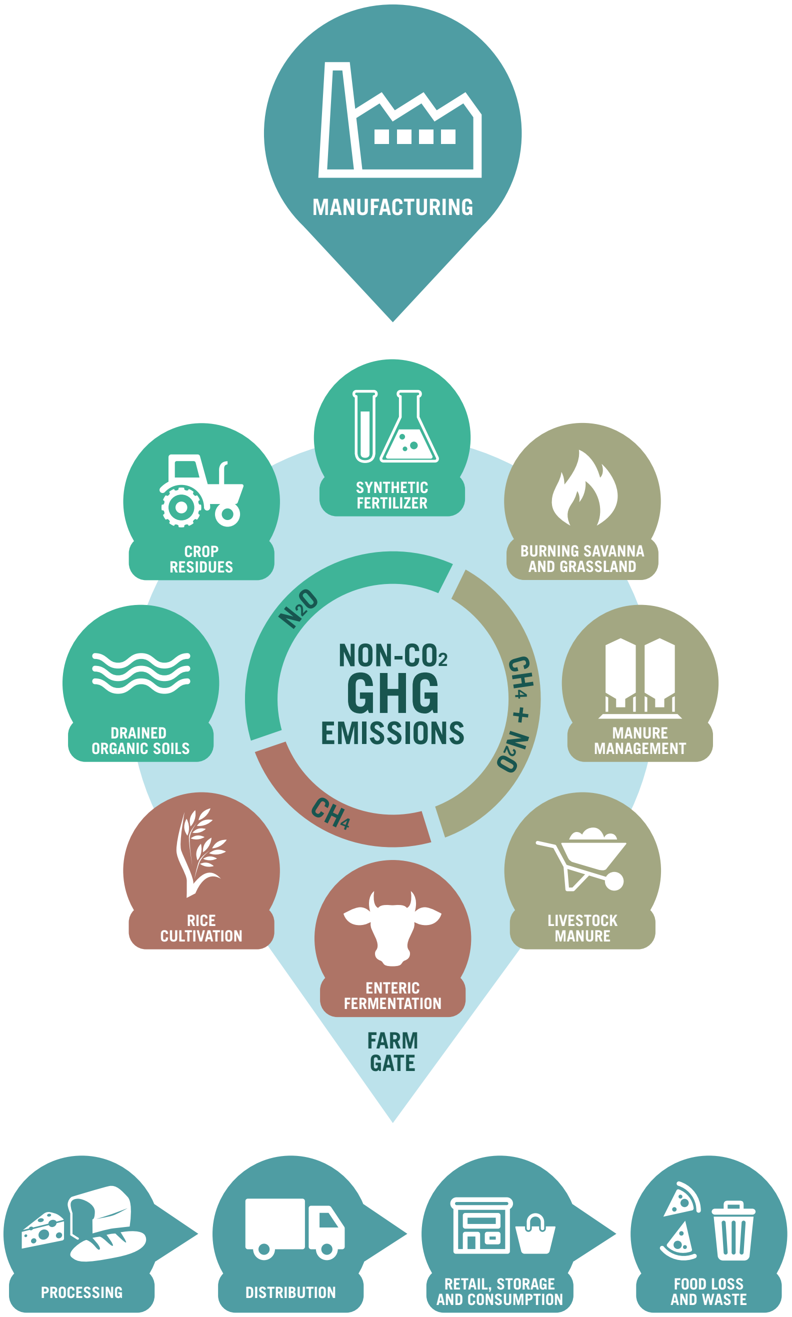
[1] sdgs.un.org/goals

[2] unfccc.int/sites/default/files/english_paris_agreement.pdf

[3] fao.org/events/detail/fao-global-conference-on-sustainable-livestock-transformation/en

FIGURE 1. **MAIN SOURCES OF NON-CO_2 GHG EMISSIONS FROM CROP AND LIVESTOCK SECTORS AND ASSOCIATED LAND USE**



The extent of contribution from upstream and downstream chain beyond the farm gate, such as manufacturing of fertilizers, industrial food processes, energy use or food loss and waste, is uncertain due to insufficient data available.

Source: Author's own elaboration. Adapted from FAO. 2020. *Emissions due to agriculture. Global, regional and country trends 2000–2018.* FAOSTAT Analytical Brief Series No 18. Rome https://www.fao.org/3/cb3808en/cb3808en.pdf and Mbow, C., Rosenzweig, C., Barioni, L.G., Benton, T.G., Herrero, M., Krishnapillai, M., Liwenga, E. *et al.* 2019. Food Security Supplementary Material. In: P.R. Shukla *et al. Climate Change and Land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems.*

BOX 1. THE ROLE OF MANURE MANAGEMENT IN REDUCING CH_4 EMISSIONS AND NUTRIENT LOSS

Greenhouse gas emissions and release of nutrients from the livestock sector can be considerably reduced through manure management strategies (FAO, 2023). Those may involve measures to reduce livestock urinary nitrogen (e.g. animal breeding for improved nitrogen efficiency, dietary manipulation), treatment of manure in digesters or composting for decreased methane (CH_4) emission, or use of urease inhibitors to reduce nitrous oxide (N_2O) emission (Montes *et al*., 2013; Rivera and Chará, 2021). These strategies are selected depending mainly on animal species, farming systems, livestock diet and climatic zone (Gerber *et al*., 2013). Various well-established treatment as well as novel processes and technologies can be employed to reduce the concentrations of substances that may affect plant growth when untreated manure is applied on land or to recover nutrients that could potentially be spread to adjacent ecosystems (Che *et al*., 2021; Dadrasnia *et al*., 2021; Szogi, Vanotti and Ro, 2015).

Notes:

Che, X., Di, H.J., Cameron, K.C. & Dodd, R. 2022. Treating farm dairy effluent with poly-ferric sulphate dramatically reduces phosphorus and *E. coli* leaching through subsurface drains—A physical drainage model study. *Soil Use and Management*, 38(3): 1493–1504. https://doi.org/10.1111/sum.12809

Dadrasnia, A., De Bona Muñoz, I., Yáñez, E.H., Lamkaddam, I.U., Mora, M., Ponsá, S., Ahmed, M. *et al*. 2021. Sustainable nutrient recovery from animal manure: A review of current best practice technology and the potential for freeze concentration. *Journal of Cleaner Production*, 315: 128106. https://doi.org/10.1016/j.jclepro.2021.128106

FAO. 2023. *Reducing methane emissions in livestock systems in Asia and the Pacific – Enhancing national climate actions through the Global Methane Pledge*. Workshop report. Bangkok, Thailand 24–26 October 2022. FAO Animal Production and Health Reports, No. 19. Rome https://doi.org/10.4060/cc6388en

Gerber, P.J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A. & Tempio, G. 2013. *Tackling climate change through livestock – A global assessment of emissions and mitigation opportunities.* Food and Agriculture Organization of the United Nations (FAO), Rome. fao.org/3/i3437e/i3437e.pdf

Montes, F., Meinen, R., Dell, C., Rotz, A., Hristov, A.N., Oh, J., Waghorn, G. *et al*. 2013. SPECIAL TOPICS — Mitigation of methane and nitrous oxide emissions from animal operations: II. A review of manure management mitigation options. *Journal of Animal Science*, 91(11): 5070–5094. https://doi.org/10.2527/jas.2013-6584

Rivera, J.E. & Chará, J. 2021. CH_4 and N_2O Emissions From Cattle Excreta: A Review of Main Drivers and Mitigation Strategies in Grazing Systems. *Frontiers in Sustainable Food Systems*, 5: 657936. https://doi.org/10.3389/fsufs.2021.657936

Szogi, A.A., Vanotti, M.B. & Ro, K.S. 2015. Methods for Treatment of Animal Manures to Reduce Nutrient Pollution Prior to Soil Application. *Current Pollution Reports*, 1(1): 47–56. https://doi.org/10.1007/s40726-015-0005-1

SCOPE AND SEARCH STRATEGY

This publication provides an overview of substances used as environmental inhibitors and summarizes information on possible food safety implications resulting from their application. In addition, the report also gives a snapshot of national and regional legislative frameworks, highlighting differences in regulating the use of environmental inhibitors in jurisdictions worldwide.

The publications cited in this document were retrieved from open-access databases such as PubMed and Google Scholar, as well as FAO' repository, using keywords such as *methanogenesis/methane inhibitor(s) or (de)nitrification/nitrogen inhibitor(s) or urease inhibitor(s) and livestock/ruminants and/or soil and/or food and/or feed and/or milk/meat/vegetable(s)/crops and/or residue(s) and/or safety and/or toxicity*.

Information and data regarding food safety of described substances were also obtained from reports, scientific opinions and institutional websites of national authorities, international bodies and associations. The regulatory section (section 4) was compiled for selected countries as representative of different geographic areas based on relevant local legislation and information provided by national authorities.

It is outside the scope of this publication to provide an exhaustive list of environmental inhibitors or information on their efficiency and application approaches. Similarly, an in-depth analysis of legislations covering environmental inhibitors in various jurisdictions is also beyond the scope of this publication.

1.1 METHANE EMISSIONS AND METHANOGENESIS INHIBITION

Methane (CH_4) is the second most abundant human-induced GHG after carbon dioxide (CO_2), (see **Box 2**) (FAO, 2020; USEPA, 2022a), and is formed as a by-product of plant biomass enteric fermentation in the digestive system of ruminants and anaerobic decomposition of the organic matter in manure or water-saturated soils (OECD, 2019). It constitutes the biggest share of GHG emissions from the livestock sector, followed by nitrous oxide (N_2O) and CO_2 (Gerber *et al.*, 2013). Microbial-derived enteric fermentative processes in ruminant livestock are responsible for about 30 percent of the total anthropogenic CH_4 emissions (FAO, 2023a and FAO, 2023b) with cattle being the major contributor to livestock emissions (see **Figure 2**) (FAO, 2023a, Gerber *et al.*, 2013).

GHG emissions have been considered as the main challenge for sustainability in the dairy sector (FAO, 2023b). Reduction of enteric CH_4 emissions is therefore necessary to limit the carbon footprint of ruminant production. Rice cultivation is the second highest source of CH_4 emissions from agrifood systems (see **Figure 2**) (IPCC, 2014; USEPA, 2022a).

Besides the negative impact of CH_4 emissions by livestock ruminants on climate change, its release is associated with reduced feed efficacy and loss of energy leading to decreased animal productivity of up to 12 percent (García-Lozano *et al.*, 2017).

Several publications offer a review of measures to mitigate CH_4 production by livestock (Almeida and Hegarty, 2021; Beauchemin *et al.*, 2020 and 2022; Cummins *et al.*, 2022; Hristov *et al.*, 2013a; Knapp *et al.*, 2014; Kumar *et al.*, 2014; Llonch *et al.*, 2017; Patra, 2011; Thompson and Rowntree, 2020). These measures include management and breeding strategies, manipulation of rumen microbiome and fermentation, vaccines, early life programming, nutritional approaches and feed supplementation. Numerous reviews focused on the latter two which can be easily introduced anytime during the animal lifespan and are part of routine husbandry practices (Black, Davison and Box, 2021; FAO, 2023a; Hadipour *et al.*, 2021; Haque, 2018; Hegarty *et al.*, 2021; Honan *et al.*, 2021; Hristov *et al.*, 2013b and 2022; Króliczewska, Pecka-Kiełb and Bujok, 2023; Palangi *et al.*, 2022; Palangi and Lackner, 2022; Sun *et al.*, 2021; Tseten *et al.*, 2022). While interventions to increase feed digestibility are associated with lower enteric CH_4 emissions, synthetic methanogenesis inhibitors used as feed additives remain more

BOX 2. CH_4 LIFESPAN AND WARMING POTENTIAL RELATIVE TO CO_2

When released to the atmosphere, methane (CH_4) immediately traps heat, at least one hundred times as much as carbon dioxide (CO_2) emissions. The warming effect diminishes as CH_4 starts to break down until cycling out in approximately twelve years. In contrast, CO_2 can persist in the atmosphere for thousands of years (USEPA, 2022; NASA, 2019). The gap between the warming effects of the two GHGs diminishes progressively: the original amount of atmospheric CH_4 would trap about 84-86 times as much heat as CO_2 over 20 years and up to 34 times over 100 years (UNECE, 2023).

The amount of GHGs is commonly expressed as CO_2 equivalents (CO_2-eq) with reference to CO_2 emissions that would cause the same time-integrated radiative forcing over a given period (Gerber *et al.*, 2013). It is obtained by multiplying a GHG emission by its global warming potential (GWP) and constitutes a standard metric for comparing emissions of different GHGs (IPCC, 2007).

Notes:

Gerber, P.J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A. & Tempio, G. 2013. *Tackling climate change through livestock – A global assessment of emissions and mitigation opportunities.* Food and Agriculture Organization of the United Nations (FAO), Rome. fao.org/3/i3437e/i3437e.pdf

Intergovernmental Panel on Climate Change (IPCC). 2007. *Climate Change 2007: Mitigation of Climate Change.* Contribution of Working Group III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. B. Metz, O.R. Davidson, P.R. Bosch, R. Dave & L.A. Meyer, eds. Cambridge University Press, Cambridge, UK and New York, USA. ipcc.ch/site/assets/uploads/2018/03/ar4_wg3_full_report-1.pdf

National Aeronautics and Space Administration of the United States (NASA). 2019. The atmosphere: Getting a Handle on Carbon Dioxide. In: *Global Climate Change, Vital Signs of the Planet.* Pasadena, USA. [Cited 07 July 2023]. climate.nasa.gov/news/2915/the-atmosphere-getting-a-handle-on-carbon-dioxide/

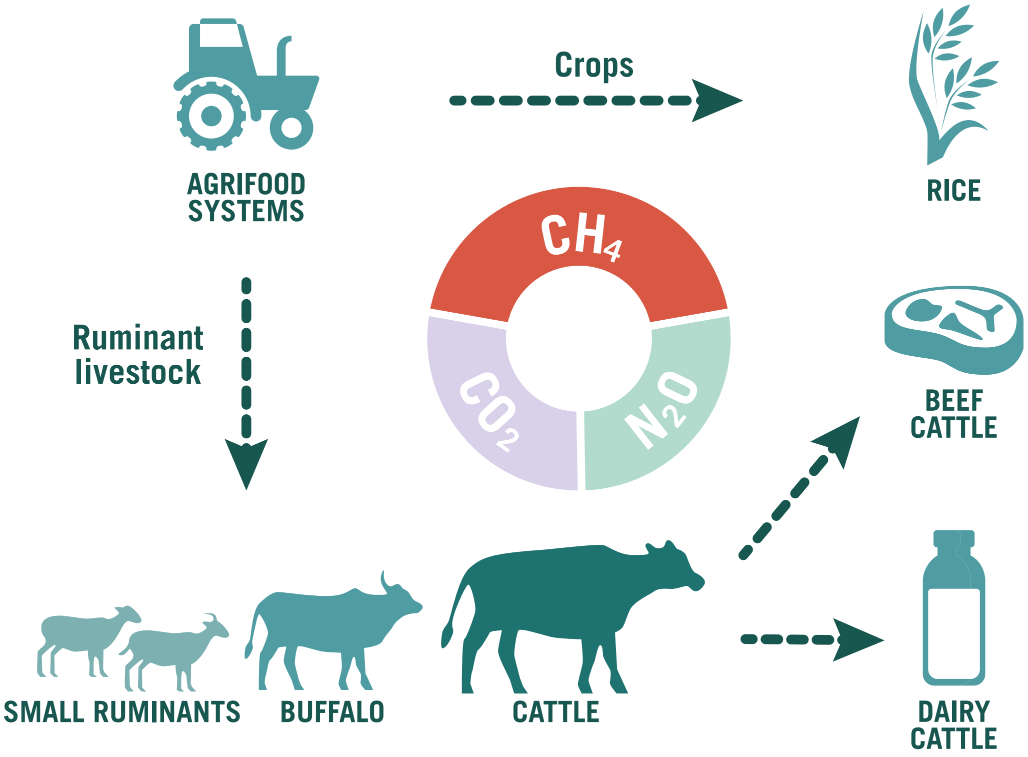
United Nations Economic Commission for Europe (UNECE). 2023. Methane management – The challenge. In: *Sustainable energy.* Geneva, Switzerland. [Cited 17 July 2023]. unece.org/challenge

United States Environmental Protection Agency (USEPA). 2022. Greenhouse gasses. In: *Climate change indicators.* Washington, DC, USA. [Cited 19 July 2023]. epa.gov/climate-indicators/greenhouse-gases

efficient and have been included among climate-smart agricultural practices (Arndt *et al.*, 2021; Veneman *et al.*, 2016; Zaman *et al.*, 2021). However, grazing and mixed farming systems need adequate solutions for the delivery of methanogenesis inhibitors to achieve substantial levels of CH_4 mitigation, such as slow-release bolus capsules releasing an inhibitor continuously at a low dose rate (NZGAGRC/PGgRc, 2021). The growing importance of methanogenesis inhibitors for the agricultural sector has also been recognized in a FAO report launched in September 2023 (FAO, 2023a).

Methanogenesis inhibitors reduce energy loss resulting from the ruminal transformation of feed nutrients into CH_4 (see **Box 3**), which limits its release from livestock to the atmosphere, and favour the production of beneficial compounds, such as fatty acids (Liu *et al.*, 2011). Methanogenesis inhibitors may also slow down methanogenic microbial processes and reduce CH_4 emission from livestock manure or water-saturated soils like rice paddies or wetlands, where decomposition of organic matter under anaerobic conditions leads to the release of considerable amounts of GHG.

FIGURE 2. **GLOBAL CH_4 EMISSIONS WITH EMPHASIS ON AGRIFOOD SYSTEMS AND LIVESTOCK**



Cattle are the major contributor with similar distribution of CH_4 emissions among beef and dairy cattle, followed by rice cultivation and other ruminants. Non-ruminant animals, such as pigs, also produce CH_4 but at much lower amounts. To a minor extent, the livestock sector is responsible for emissions of other GHGs, such as N_2O and CO_2.

Source: Author's own elaboration. Adapted from FAO. 2023. *Methane emissions in livestock and rice systems.* Rome. https://doi.org/10.4060/cc7607en; Gerber, P.J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A. & Tempio, G. 2013. *Tackling climate change through livestock – A global assessment of emissions and mitigation opportunities.* Food and Agriculture Organization of the United Nations (FAO), Rome. fao.org/3/i3437e/i3437e.pdf; IPCC, 2014. *Climate Change 2014: Synthesis Report.* Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]; IPCC, Geneva, Switzerland, 151 pp. and USEPA. 2022a. *Importance of Methane.* In: Global methane initiative. Washington, DC, USA. [Cited 19 July 2023]. epa.gov/gmi/importance-methane

Methanogenesis inhibitors specifically target microorganisms responsible for the production of CH_4 by acting on enzymes which are not present in non-methanogenic species. The most widely described compounds are analogues of methyl-coenzyme M (Me-CoM), which is the substrate of methyl coenzyme M reductase (MCR) enzyme. MCR is responsible for catalysing the last step in the process of CH_4 production by methanogenic archaea. Methanogenesis inhibitors competitively inhibit MCR by binding to the enzyme's active site, which impedes binding of the natural substrate and hampers the reaction (see **Figure 3**). Recently,

Rice paddies emit CH_4 due to large volumes of water necessary to sustain plant growth, which blocks oxygen from penetrating the soil. This generates optimal conditions for growth of methanogenic bacteria.

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the Me-CoM analogue 3-nitrooxypropanol (3-NOP) has gained attention due to its efficacy in enteric CH_4 inhibition and positive safety evaluation by the producer and food safety authorities, supported by numerous reviews describing its properties and use as feed additive (Jayanegara *et al.*, 2017; Kebreab *et al.*, 2023; Kim *et al.*, 2020; Yu, Beauchemin and Dong, 2021). Food safety details are provided in section 2.1.1.1. However, methanogenesis inhibitors can also target other enzymes, such as 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA), which is involved in the synthesis of cell membrane components in archaea and can be inhibited by statins (Gottlieb *et al.*, 2016).

BOX 3. METHANOGENESIS PROCESSES AND TARGETS OF METHANOGENESIS INHIBITORS

Complex organic matter taken up with feed is reduced to less complex matter in the process of microbial hydrolysis carried out by bacteria, fungi and protozoa (Cammack *et al.*, 2018). Then, microorganisms responsible for fermentation produce short-chain volatile fatty acids (VFA) such as acetate, propionate and butyrate which are absorbed for nutrition and for the production of meat, milk and wool (Ungerfeld, 2020).

Carbon dioxide (CO_2) and hydrogen (H_2) are by-products of fermentation and can be used by hydrogenotrophic methanogens to produce methane (CH_4) as the primary route of methanogenesis. In this process, binding of enzyme Me-CoM reductase (MCR) with its natural substrate methyl coenzyme M (Me-CoM) is crucial and can be blocked by MCR inhibitors (Yu, Beauchemin and Dong, 2021).

As animals have a fast flow of organic matter, rumen methanogenesis is partial (Leahy *et al.*, 2022); in ecosystems such as rice paddies, VFA can be converted to simpler acids while the process of acetogenesis leads to the production of acetic acid (CH_3COOH) from CO_2 and H_2, which is further transformed by acetoclastic methanogens with a final release of CH_4. Accumulated gas is eliminated primarily during eructation and respiration.

In addition to MCR inhibitors, different reactions and organisms along the pathway are targets of other inhibitors to reduce CH_4 emissions. Inhibitors of hydrogenotrophic and acetoclastic methanogens modulate the respective populations limiting their activity, thereby reducing the amount of produced CH_4. At the very beginning of the digestive process, the population of protozoa can be kept under control by bioactive compounds contained in biological methanogenesis inhibitors, which may also inhibit hydrogenotrophic and acetoclastic methanogens. Alternative H_2 sinks reduce available hydrogen produced during microbial activity subtracting it from methanogens, thereby reducing the amount of CH_4 produced.

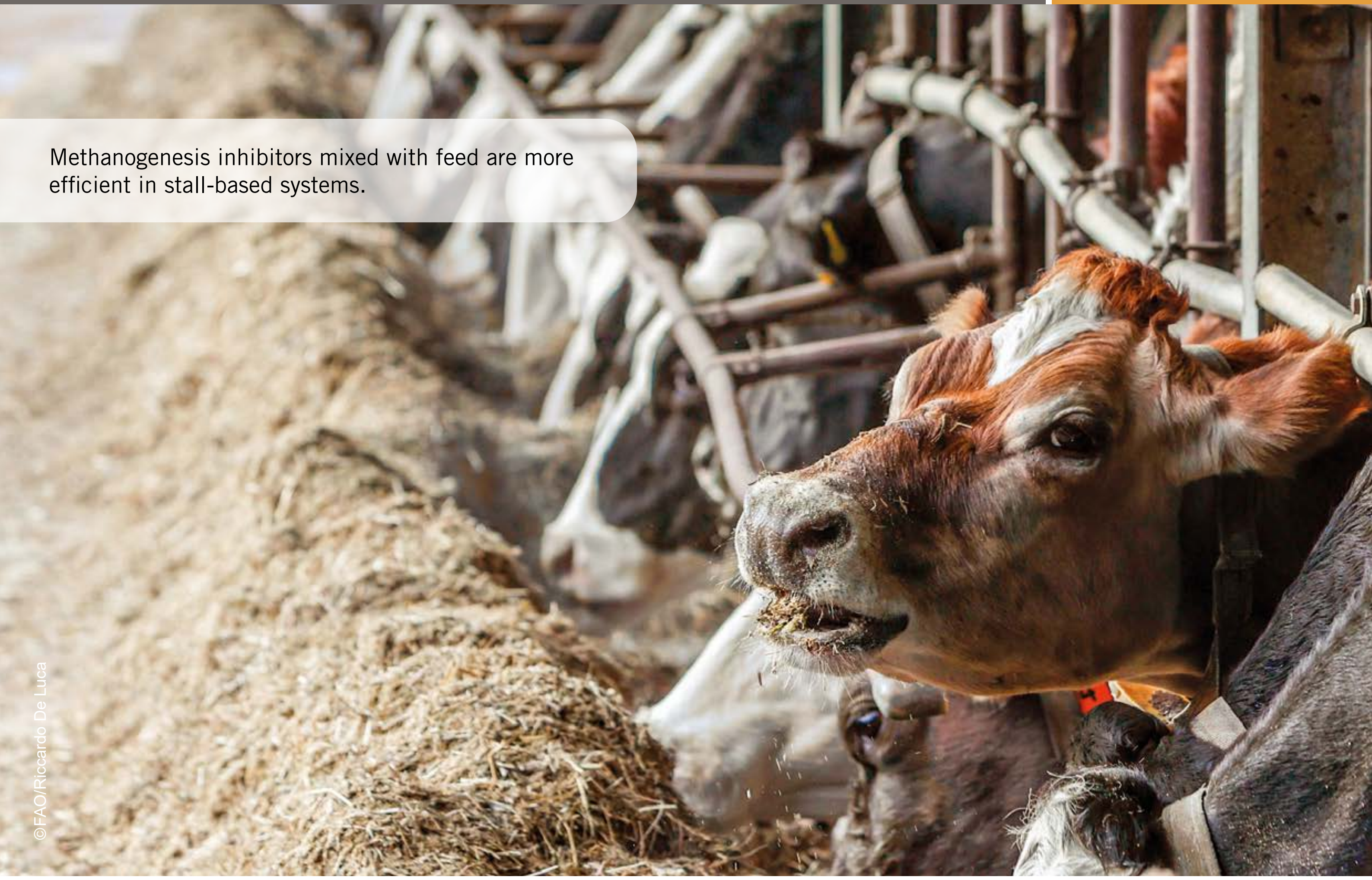
Notes:

Cammack, K.M., Austin, K.J., Lamberson, W.R., Conant, G.C. & Cunningham, H.C. 2018. Tiny but mighty: The role of the rumen microbes in livestock production. *Journal of Animal Science*. https://doi.org/10.1093/jas/skx053

Leahy, S.C., Janssen, P.H., Attwood, G.T., Mackie, R.I., McAllister, T.A. & Kelly, W.J. 2022. Electron flow: key to mitigating ruminant methanogenesis. *Trends in Microbiology*, 30(3): 209–212. https://doi.org/10.1016/j.tim.2021.12.005

Ungerfeld, E.M. 2020. Metabolic Hydrogen Flows in Rumen Fermentation: Principles and Possibilities of Interventions. *Frontiers in Microbiology*, 11: 589. https://doi.org/10.3389/fmicb.2020.00589

Yu, G., Beauchemin, K.A. & Dong, R. 2021. A Review of 3-Nitrooxypropanol for Enteric Methane Mitigation from Ruminant Livestock. *Animals*, 11(12): 3540. https://doi.org/10.3390/ani11123540



Methanogenesis inhibitors mixed with feed are more efficient in stall-based systems.

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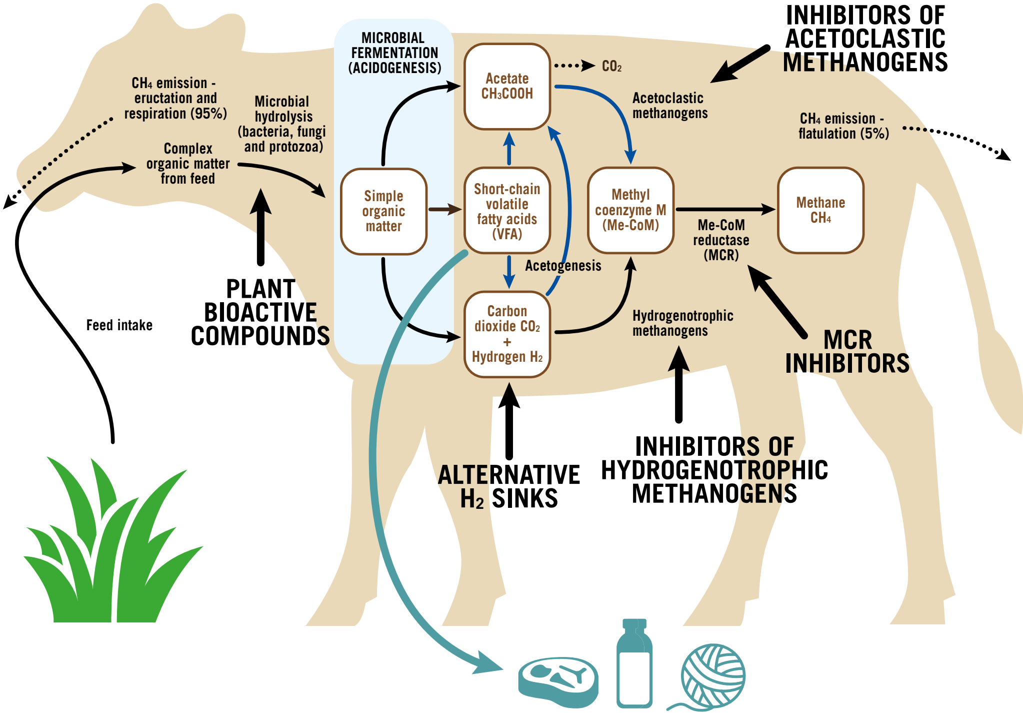
Non-specific methanogenesis inhibitors not only target microorganisms directly involved in the production of methane but also inhibit wider microbial populations by acting on common processes or molecules (Liu *et al.*, 2011). An example of this type of inhibitors are ionophores used in animal production as antimicrobials.

These can improve feed efficiency by modulating rumen microorganisms and fermentation pathways leading to shifts in the production of volatile fatty acids (VFA) from acetate to propionate. This contributes to reducing CH_4 emissions by lowering the amount of substrate for methanogenic bacteria. The mode of action of ionophores as feed additives and their impact on ruminal function has been reviewed previously (da Silva Marques and Fernandes Cooke, 2021; FAO, 2023a; Hall, 2013).

Several substances acting as hydrogen sinks alternative to CH_4 have been identified (Newbold *et al.*, 2005). These act by competing with CH_4 for available hydrogen, impeding the reaction with CO_2 through hydrogenotrophic route (see **Figure 3**).

However, only few substances (e.g. nitrates) proved to efficiently trap hydrogen and reduce methanogenesis, which may be explained by additional mechanisms involved (Yang *et al.*, 2016). For example, in an endogenous process in the rumen, nitrites are produced as intermediates from nitrates following reduction by hydrogen. In turn, nitrites may alter microbial metabolism lowering the amount of generated hydrogen or inhibit methanogens directly (Ungerfeld, 2015; Yang *et al.*, 2016).

FIGURE 3. MAIN STEPS OF CH_4 GENERATION PATHWAY IN RUMINANTS AND POINTS OF INTERVENTION TO REDUCE CH_4 EMISSIONS


MICROBIAL FERMENTATION (ACIDOGENESIS)
INHIBITORS OF ACETOCLASTIC METHANOGENS
CH_4 emission - eructation and respiration (95%)
Microbial hydrolysis (bacteria, fungi and protozoa)
Complex organic matter from feed
Acetate CH_3COOH
CO_2
Acetoclastic methanogens
CH_4 emission - flatulation (5%)
Simple organic matter
Short-chain volatile fatty acids (VFA)
Methyl coenzyme M (Me-CoM)
Methane CH_4
Me-CoM reductase (MCR)
Acetogenesis
Carbon dioxide CO_2 + Hydrogen H_2
Hydrogenotrophic methanogens
Feed intake
PLANT BIOACTIVE COMPOUNDS
MCR INHIBITORS
ALTERNATIVE H_2 SINKS
INHIBITORS OF HYDROGENOTROPHIC METHANOGENS

Processes prevailing in gut ecosystems (ruminants, humans) are indicated by black arrows while those prevailing in environmental ecosystems are shown by blue arrows.

Source: Author's own elaboration. Adapted from Haque, M.N. 2018. Dietary manipulation: a sustainable way to mitigate methane emissions from ruminants. *Journal of Animal Science and Technology*, 60(1): 15. https://doi.org/10.1186/s40781-018-0175-7; Leahy, S.C., Janssen, P.H., Attwood, G.T., Mackie, R.I., McAllister, T.A. & Kelly, W.J. 2022. Electron flow: key to mitigating ruminant methanogenesis. *Trends in Microbiology*, 30(3): 209–212. https://doi.org/10.1016/j.tim.2021.12.005; Liu, H., Wang, J., Wang, A. & Chen, J. 2011. Chemical inhibitors of methanogenesis and putative applications. *Applied Microbiology and Biotechnology*, 89(5): 1333–1340. doi.org/10.1007/s00253-010-3066-5; Ungerfeld, E.M. 2020. Metabolic Hydrogen Flows in Rumen Fermentation: Principles and Possibilities of Interventions. *Frontiers in Microbiology*, 11: 589. https://doi.org/10.3389/ fmicb.2020.00589 and Yu, G., Beauchemin, K.A. & Dong, R. 2021. A Review of 3-Nitrooxypropanol for Enteric Methane Mitigation from Ruminant Livestock. *Animals*, 11(12): 3540. https://doi. org/10.3390/ani11123540

Biological methanogenesis inhibitors include a variety of feed additives usually based on products containing mixtures of active substances. Some species of seaweed such as *Asparagopsis taxiformis* and *Asparagopsis armata*, have showed high inhibitory potential, reducing up to 98 percent of ruminal CH_4 emissions (Bačėninaitė, Džermeikaitė and Antanaitis, 2022). Seaweeds have halogenated CH_4 analogues (HMAs) which can block the production of Me-CoM necessary for the last step of methanogenesis by reacting with vitamin B_{12} and impeding cobamide-dependent methyl transfer into coenzyme M. Other seaweeds with lower concentrations of HMAs can inhibit methanogenesis through a variety of bioactive components but with lower efficacy compared to *Asparagopsis* spp. However, many of the studies on seaweed inclusion for CH_4 reduction are based on in vitro, short-term experiments with inconsistent results, albeit mostly indicating a reduction in CH_4 output (FAO, 2023a; Lean *et al.*, 2021).

While various plant-derived compounds including saponins, tannins, flavonoids and essential oils have been studied for their properties to inhibit CH_4 emission from livestock (FAO, 2023a), these have mainly been used to improve the nutritional characteristics of feed and food.

1.2 NITROGEN LOSS AND ITS INHIBITION

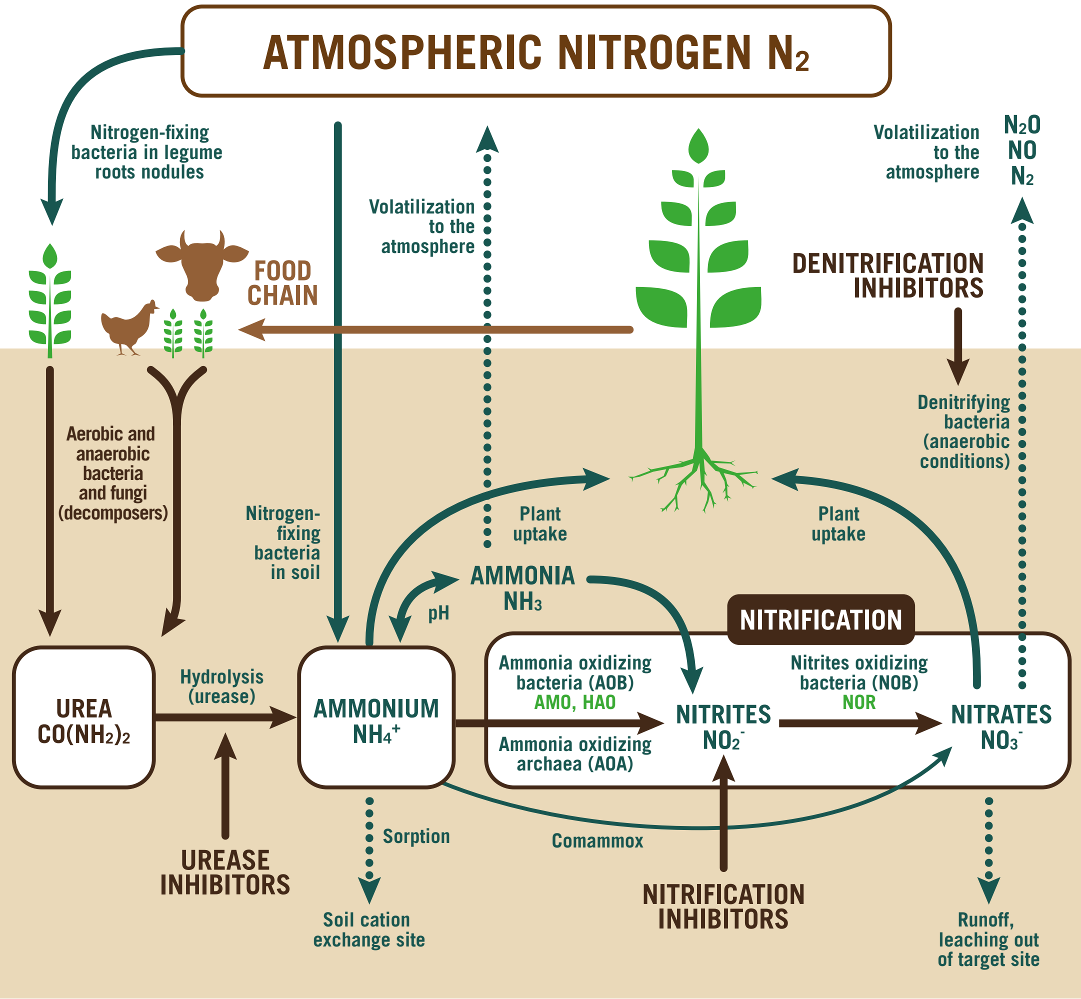
Nitrogen is critical for optimal growth and development of plants, ensuring efficient crop production. Despite being one of the most abundant elements in nature, lack of nitrogen is the main cause of nutritional deficiency in plants (Pallardy, 2008). Among plant nutrients, nitrogen is the one in highest demand. Over 50 percent of fertilizers is produced as urea which has a high nitrogen concentration and lower production costs compared to other nitrogen fertilizers (Cantarella *et al.*, 2018; Cross, L. and Gruère, 2022). Urea is also naturally present in animal excreta. When applied to soil, urea undergoes fast hydrolysis to ammonium which plants can utilize for growth. However, ammonia and other reactive nitrogen compounds, such as nitrates, can be formed from ammonium (see **Figure 4**), leading to the loss of even half of nitrogen input following fertilization (Lassaletta *et al.*, 2014). Those processes depend on multiple factors including climatic conditions, soil and crop type, agronomic practices, site position in the landscape, and nitrogen concentration (Robertson, 1997).

The loss of nutrients resulting from their chemical properties and natural dynamics in soil (see **Figure 4**) will need to be managed to restore ecosystem (Hunter *et al.*, 2017). This imbalance relates to both chemical and organic fertilizers which can lead to excessive nutrient input or their deficiency in soil if used improperly (Shaji, Chandran and Mathew, 2021). Various mitigation measures exist to manage the loss of nitrogen from soil in relation to agricultural land use (Abalos *et al.*, 2022; Hassan *et al.*, 2022; Hoekstra *et al.*, 2020; Xia *et al.*, 2017). Among them, nitrogen inhibitors reduce the transformation of nitrogen compounds into less stable forms prone to leaching or volatilization (see **Box 4**). This allows the available nitrogen to be stabilized on the soil particles and provides plants with increased access to nitrogen.

The characteristics, modes of action, efficacy and strategies of employment have been widely described in numerous reviews on nitrification (Beeckman, Motte and Beeckman, 2018; Fan *et al.*, 2022; Nugrahaeningtyas *et al.*, 2022; Ray *et al.*, 2020; Woodward *et al.*, 2021) and urease inhibitors (Cantarella *et al.*, 2018; Klimczyk, Siczek and Schimmelpfennig, 2021; Modolo *et al.*, 2018; Song *et al.*, 2022; Yang *et al.*, 2022; Yang, Peng and Wang, 2023), many of which focus on biological solutions (Coskun *et al.*, 2017a and 2017b; Modolo *et al.*, 2015; Nardi *et al.*, 2020; Sadhukhan *et al.*, 2022; Saud, Wang and Fahad, 2022; Subbarao *et al.*, 2013a, 2013b and 2015; Wang, X. *et al.*, 2021). By improving nitrogen use efficiency (NUE) in plants, smaller amounts of fertilizers are required to maintain efficiency of food production.

Consequently, leaching of fertilizers to groundwater and surface waters is reduced resulting in beneficial effects on eutrophication (Singh and Verma, 2008). Precision agriculture solutions to reduce leaching of nitrate and emissions of ammonia and nitrous oxide are also under development. Solutions for grazing systems involve robotics to simultaneously detect and treat urine patches with nitrogen inhibitors (Bates, Quin and Bishop, 2015).

FIGURE 4. MAIN PATHWAYS OF BIOLOGICAL NITROGEN FIXATION, LOSS AND STEPS ON WHICH NITROGEN INHIBITORS ACT


ATMOSPHERIC NITROGEN N_2
Nitrogen-fixing bacteria in legume roots nodules
Volatilization to the atmosphere
Volatilization to the atmosphere
N_2O
NO
N_2
FOOD CHAIN
DENITRIFICATION INHIBITORS
Denitrifying bacteria (anaerobic conditions)
Aerobic and anaerobic bacteria and fungi (decomposers)
Nitrogen-fixing bacteria in soil
Plant uptake
Plant uptake
AMMONIA NH_3
pH
NITRIFICATION
UREA $CO(NH_2)_2$
Hydrolysis (urease)
AMMONIUM NH_4^+
Ammonia oxidizing bacteria (AOB)
AMO, HAO
Ammonia oxidizing archaea (AOA)
NITRITES NO_2^-
Nitrites oxidizing bacteria (NOB)
NOR
NITRATES NO_3^-
UREASE INHIBITORS
Sorption
Soil cation exchange site
Comammox
NITRIFICATION INHIBITORS
Runoff, leaching out of target site

Source: Author's own elaboration. Adapted from Bernhard, A. 2010. The Nitrogen Cycle: Processes, Players, and Human Impact. *Nature Education Knowledge*, 3(10):25. nature.com/scitable/knowledge/library/the-nitrogen-cycle-processes-players-and-human-15644632/; Norton, J. & Ouyang, Y. 2019. Controls and Adaptive Management of Nitrification in Agricultural Soils. *Frontiers in Microbiology*, 10: 1931. https://doi.org/10.3389/fmicb.2019.01931 and Wagner, S. C. 2011. Biological Nitrogen Fixation. *Nature Education Knowledge*, 3(10):15. nature.com/scitable/knowledge/library/biological-nitrogen-fixation-23570419/

BOX 4. MAIN PROCESSES OF NITROGEN CYCLE IN THE SOIL RELEVANT FOR NITROGEN INHIBITORS

As atmospheric nitrogen can be used only by legume plants thanks to symbiotic bacteria living in their roots, nitrogen fixation is necessary for organisms to produce vital organic compounds such as proteins or nucleic acids (Wagner, 2011). Through their roots, plants assimilate nitrogen in the form of ammonium and nitrates which are dissolved in water present in soil. Once fixed in the vegetal mass, nitrogen is distributed in the food chain to other organisms like animals. Organic waste is then decomposed to urea by aerobic and anaerobic bacteria and fungi residing in soil.

The overall pool of nitrogen in the form of urea can be increased using fertilizers. In the presence of water, the process of hydrolysis mediated by urease enzyme leads to the production of ammonium from urea and is the first point in the pathway where nitrogen inhibitors can be applied (urease inhibitors). Ammonium can be also produced from atmospheric nitrogen by specialized soil bacteria, and it can be stabilized onto soil on negatively charged exchange sites. However, it can be lost through volatilization of ammonia which is formed under alkaline conditions, and during nitrification. Urease inhibitors reduce this loss by limiting the hydrolysis of urea, primarily by inhibiting ammonia monooxygenase (AMO), which gives time for its incorporation into the soil via rainfall or irrigation, and allows the formation of ammonia at lower rates compatible with plant uptake.

Both ammonium and ammonia undergo a rapid process of nitrification to nitrites carried out by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA), and in the second step to nitrates by nitrites oxidizing bacteria (NOB) (Norton and Ouyang, 2019). Comammox (complete ammonia oxidation) bacteria belonging to the genus Nitrospira can perform both conversion processes. Nitrates can rapidly accumulate in the soil and be lost by runoff, leaching out to depths below the rhizosphere and farther to the environment, or via a conversion to atmospheric nitrogen by denitrifying bacteria under anaerobic conditions (Bernhard, 2010). This loss can be limited by applying nitrification inhibitors which temporarily reduce the activity of Nitrosomonas (included among AOB) and Nitrobacter bacteria (included among NOB) in soil or enzymes produced by them, such as AMO, hydroxylamine oxidoreductase (HAO) or nitric oxide reductase (NOR). In particular, sandy soils which are not capable of withholding nitrates as well as wet soils where ammonium attached to the soil particles is easily washed out are the most suitable candidates for the application of nitrification inhibitors. Additionally, by-products of denitrification, nitrous oxide (N_2O) and nitric oxide (NO), contribute respectively to GHG emission and to smog. Both phenomena can be limited by applying denitrification inhibitors which target the activity of denitrifying bacteria.

Notes:

Bernhard, A. 2010. The Nitrogen Cycle: Processes, Players, and Human Impact. *Nature Education Knowledge*, 3(10):25. nature.com/scitable/knowledge/library/the-nitrogen-cycle-processes-players-and-human-15644632/

Norton, J. & Ouyang, Y. 2019. Controls and Adaptive Management of Nitrification in Agricultural Soils. *Frontiers in Microbiology*, 10: 1931. https://doi.org/10.3389/fmicb.2019.01931

Wagner, S. C. 2011. Biological Nitrogen Fixation. *Nature Education Knowledge*, 3(10):15. nature.com/scitable/knowledge/library/biological-nitrogen-fixation-23570419/

Environmental inhibitors can help improve the efficacy of nitrogen use in crops, making it possible to decrease the required amounts of fertilizers.

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CHAPTER 2

FOOD SAFETY OF ENVIRONMENTAL INHIBITORS

While environmental inhibitors have been studied for decades, research to date has mainly focused on evaluating their efficacy in reducing CH_4 emissions and improving the productivity of agrifood systems. Effects on target organisms are widely described, but much less has been published on the impact that environmental inhibitors may have on non-target organisms as well as on humans through consumption of contaminated foods. Information on potential residues of environmental inhibitors (and their metabolites) in food commodities is sparse and the related food safety risks have rarely been assessed.

This chapter explores whether residues of environmental inhibitors found in animal products, crops and vegetables might cause food safety concerns. The following sections describe environmental inhibitors grouped according to their chemical structure or source, providing summaries of safety- related information, where available.

For the purpose of this publication, the term 'synthetic inhibitors' refers to those inhibitory substances which are generally obtained through industrial synthesis processes. For these inhibitors, substances have been selected as examples of various chemical classes, thereby having potentially different modes of action and toxicity effects. Constituents of commercial products currently on the market are included under this term. The term 'biological inhibitors' indicates inhibitory compounds, their mixtures or parts derived from natural sources (e.g. plants or seaweeds). This term aligns to denomination commonly used in scientific literature. Biological inhibitors can be constituted by mixtures of chemically different substances, therefore descriptions in this publication are based on their natural origin.

While food safety is the focus of this section, pertinent information could not be identified in some cases. Although outside the scope of this work, effects on the environment, including toxicity in wild animals, are provided as additional information even if not directly related to dietary exposure. Likewise, hazard-related information described in this section should not be considered as necessarily constituting a health risk in the absence and/or at low levels of exposure. It should also be noted that information referring to occupational exposure is based on higher concentrations than those occurring in food. Information on hazard according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) refers to pure substances, their diluted solutions or mixtures in bulk quantities.[4]

2.1 METHANOGENESIS INHIBITORS

Numerous inhibitors exist to reduce ruminal CH_4 emissions acting on different points along the methanogenesis pathway (see **Figure 3**). Whilst some of these inhibitors are effective (e.g. 2,2,2-trichloroacetamide, hemiacetal of chloral and starch, bromochloromethane, anthraquinone), they present numerous drawbacks including transient reduction in CH_4 emissions, undesirable side effects in livestock, toxicity in animals and humans, possible adaptation of the rumen to inhibitors over time or accumulation in meat. These drawbacks impede their application in agricultural practices (Beauchemin *et al.*, 2020; McGinn *et al.*, 2019; Yang *et al.*, 2016). Information on selected categories of methanogenesis inhibitors regarding safety for livestock has recently been summarized in a FAO report (FAO, 2023a). The most important substances applied as methanogenesis inhibitors, (including those currently considered as the most promising along with the ones formerly studied), are listed in **Table 1** according to type and mode of action.

2.1.1 SYNTHETIC METHANOGENESIS INHIBITORS

These inhibitors comprise single chemical substances or mixtures including the active substance and other constituents of commercial products. The chemical classes below are reported in alphabetical order within their respective types of inhibitors.

Inhibitors of MCR and other enzymes

Anthraquinone-based compounds

The group of anthraquinone-based compounds comprises natural and synthetic compounds with multiple uses, including application in human medicine, cosmetics, food packaging and as food colourants. Numerous studies reported toxicity of various substances belonging to anthraquinones group primarily on liver, kidney, or gastrointestinal tract, with DNA damage as a critical long-term effect (Health Canada, 2018; Shukla *et al.*, 2017). Low concentrations of anthraquinones naturally present in a balanced diet as well as those used in food production and consumer products are generally not considered to pose a threat to animal and human health through oral or dermal exposure, or to endanger the environment (Dufossé, 2014;

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[4] Globally Harmonised System of Classification and Labelling of Chemicals (GHS). 2021. Ninth revised edition. United Nations, New York and Geneva. ST/SG/AC.10/30/Rev.9

Livestock feed can be supplemented with methanogenesis inhibitors to reduce CH_4 emissions.

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Fouillaud *et al.*, 2018; Health Canada, 2018). Anthraquinones may form reactive metabolites and transform one into another potentially leading to an increased effect (Fouillaud *et al.*, 2018 ; Wang, D. *et al.*, 2021).

Most of the toxicological information related to the agrifood sector is available for 9,10-anthraquinone used as a pesticide. Toxicological risk to humans from the non-food outdoor uses of 9,10-anthraquinone have been considered negligible (USEPA, 1998). Residues of 9,10-anthraquinone used as bird repellent on seeds are likely in treated food commodities such as rice. However, no concern to human health through chronic dietary exposure based on rice consumption and drinking water has been identified by the United States Environmental Protection Agency (USEPA) (USEPA, 2022b). Nevertheless, potential cancer risk has been identified from residential exposure to treated turf and from occupational exposures (USEPA, 2022c). Potential adverse effects to non-target animals and aquatic plants have been identified but data are insufficient to perform risk assessment for some terrestrial taxa (USEPA, 2022b). The use of 9,10-anthraquinone as pesticide is not authorized in the European Union (EU) (EU, 2022a).

Considering the hazard properties of 9,10-anthraquinone, this compound has been classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (Group 2B) (IARC, 2013).

Chlorinated phenols

These substances can also be employed as pesticides and preservatives. A study on kinetics of methanogenesis in the presence of chlorophenols indicated that toxicity to microorganisms increases with hydrophobicity of compounds and is related to their adsorption capacity to sludge (Puyol *et al.*, 2012). Thus, pentachlorophenol (PCP) has been considered more toxic compared to less hydrophobic 2,4-dichlorophenol (2,4-DCP) and 2,4,6-trichlorophenol (2,4,6-TCP). Following the evaluation by the Joint FAO/World Health Organization (WHO) Meeting on Pesticide Residues (JMPR), exposure to PCP through diffuse sources including food and drinking water had been considered to be of very low concern for the general population, however, it may show toxicity effects to non-target species, including inhibition of growth and development, mortality or altered biomarkers (FAO/WHO, 1987).

Guidance values established by WHO for 2,4,6-TCP as disinfection by-product in drinking water are 2000, 200 and 20 µg/L for 10^{-4}, 10^{-5} and 10^{-6} excess lifetime cancer risks, respectively (WHO, 2003). The lowest reported taste threshold for 2,4,6-TCP is 2 µg/L, therefore, water free from taste has been considered unlikely to raise risk concerns for consumers. No guideline values have been derived for 2,4-DCP and 2-chlorophenol in drinking water due to limited toxicity data (WHO, 2003). 2,4-DCP may be formed as a photodegradation product of triclosan (Latch *et al.* 2005).

Concerning hazard from chlorinated phenols, PCP is classified by the IARC as carcinogenic to humans (Group 1) based on evidence to cause non-Hodgkin lymphoma in humans (IARC, 2019). It is classified by the European Chemicals Agency (ECHA) as a suspected carcinogen (ECHA, 2023) and by the USEPA as likely to be carcinogenic to humans (USEPA, n.d.a.). Therefore, based on occupational exposure, PCP is being phased out as a wood preservative in the USA (USEPA, n.d.b.). PCP is also considered hepatotoxic with a reference dose for oral exposure established at 5 µg/kg per day (USEPA, 2023). 2,4,6-TCP is classified by the IARC as possibly carcinogenic to humans (Group 2B) (IARC, 2019) and by the USEPA as a probable human carcinogen causing leukaemia based on limited or no human data (Group B2) (USEPA, n.d.b.).

Halogenated CH_4 analogues

This category of methanogenesis inhibitors has been reviewed previously (Patra *et al.* 2017). The use of halogenated compounds is not authorized in numerous countries due to concerns related to their involvement in ozone depletion (Montreal Protocol, 1987). The major route of human exposure to trihalomethane have been reported to occur through drinking water and inhalation (WHO, 2004). Chloroform evaluated by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) has been considered unsuitable for use as a food additive (FAO/WHO, 1980). The tolerable daily intake (TDI) for chloroform has been derived at 13 µg/kg body weight per day (corrected for weekly exposure of 6 days) based on hepatotoxic effects in dogs, while a guidance value for exposure through drinking water (2 L daily ingestion) has been set at 200 µg/L based on an average body weight of 60 kg (WHO, 2004). The TDI for bromoform and dibromochloromethane (DBCM) have been established at 17.9 and 21.4 µg/kg body weight per day (corrected for exposure over 5 days per week), respectively, with guideline values allocating 20 percent of the TDI to drinking water derived at 100 µg/L for both compounds (WHO, 2004).

Considering possible carcinogenicity effects, guidance values established by WHO for bromodichloromethane (BDCM) are 600, 60 and 6 µg/L for 10^{-4}, 10^{-5} and 10^{-6} excess lifetime cancer risks, respectively (WHO, 2004). It is not considered a common food contaminant, but trace amounts can be present in dairy products (WHO, 2004). Residues of bromochloromethane (BCM) in steer meat, fat and offal were detected within safety limits, however, concentrations lost due to volatilization were not calculated (Tomkins, Colegate and Hunter, 2009).

Suspected or probable/possible carcinogenicity[5] and suspected toxicity for reproduction[6] have been indicated for some compounds of this group. Classification by the IARC reports possible carcinogenic effects to humans (Group 2B) for chloroform and BDCM, while bromoform and DBCM could not be classified as to their carcinogenicity to humans due to limited evidence (IARC, 1991 and 1999).

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[5] www.epa.gov/iris

[6] echa.europa.eu

Chronic toxicity to aquatic organisms has been notified for bromoform, iodoform and carbon tetrachloride. Positive results were obtained for 2,2,2-trichloroacetamide in mammalian cytotoxicity and genotoxicity assays (Plewa *et al.*, 2009). According to the GHS, the following hazards have been associated with these substances: acute toxicity when swallowed or inhaled, skin and serious eye irritations as well as possible respiratory irritations.

Nitrooxy compounds

3-NOP has been recently commercialized as the active substance of "Bovaer® 10". Based on available absorption, distribution, metabolism and excretion (ADME) data, it has been considered safe for target species, intended as ruminants for milk production and reproduction, at maximum recommended levels in feed of 100 mg 3-NOP/kg dry matter (Almeida and Hegarty, 2021; EFSA, 2021a). Mutagenic and genotoxic effects of 3-NOP and its metabolites have not been observed in rats (Thiel *et al.*, 2019a and 2019b). Under the conditions of use as feed additive proposed by the manufacturer, safety concern for consumers and the environment have not been reported from 3-NOP nor its metabolites 3-nitrooxypropionic acid (NOPA), 1,3-propanediol and nitrate, the latter two naturally occurring in the rumen (EFSA, 2021a; FAO, 2023a). Safety for other animal species could not be estimated (EFSA, 2021a).

Other nitrooxy compounds have been studied or proposed for their ability to reduce enteric CH_4 emissions (Duval and Kindermann, 2012; Jin *et al.*, 2017; Martínez-Fernández *et al.*, 2014). However, they need further evaluation regarding side effects on animal health, residues in food commodities and safety for consumers (Li *et al.*, 2021).

Organosulfonic acids

Information on potential risks to animals and consumers from dietary sources could not be identified. Adaptation and resistance of some rumen methanogens to 2-bromoethanesulfonate (BES) has been observed suggesting subsequent increase in growth and persistence of BES-resistant microorganisms (Ungerfeld *et al.*, 2004). Understanding the mode of action of some substances, such as (4-hydroxyphenyl) chloromethanesulfonate (C-1), needs further research (Hotta *et al.*, 2022).

According to the GHS, hazard from many sulfonic esters may include (severe) skin and serious eye irritation or damage, respiratory irritation upon inhalation or oral exposure during handling. Extrapolation of this information to inform about dietary risk must be performed with caution, as concentrations of these compounds in the diet are several orders lower.

Pterins

Lumazine is the representative compound belonging to pterins which have been formerly studied for methanogenesis inhibition (Nagar-Anthal *et al.*, 1996; Ungerfeld *et al.*, 2004; Ungerfeld, Rust and Burnett, 2007). Lumazine occurs naturally in plants, insects and some marine organisms (Daniels *et al.*, 2019). Information concerning consumers' safety has not yet been collected.

Statins

While adverse effects of statins used in human medicine to control cholesterol levels are widely described, safety information on statins as feed additives with effects on consumers through residues in animal-derived food is sparse. No harmful effects on health of goats fed a diet supplemented with lovastatin was observed (Candyrine *et al.*, 2018). Likewise, rumen microbiota was not affected. When used as food supplements, the intake of lovastatin was considered safe at the use level of 10 mg/day, although sporadic effects on liver and musculoskeletal system occurred at lower concentrations (EFSA, 2018a).

Alternative H_2 sinks

Carboxylic acids

Some studies described the efficacy of propynoic acid and ethyl 2-butynoate in reducing CH_4 emission from the rumen (Ungerfeld, Rust and Burnett, 2003). Toxicity information for ruminants has not been identified for propynoic acid. However, propynoic acid is an unsaturated analogue of propionic acid which has been assessed for safety as feed additive to all species and considered not to pose safety concerns for consumers (EFSA, 2011). The acceptable daily intake (ADI) established by JEFCA is "not limited" for propionic acid used as food additive at proposed use levels (FAO/WHO, 1999). The evaluation by the European Food Safety Authority (EFSA) considered that propionic acid does not raise safety concerns from dietary exposure at concentrations up to 41.5 mg/kg body weight per day, with the use as food additive being the major contributor (EFSA, 2014a). Likewise, no concern with respect to carcinogenicity and genotoxicity was identified, although for the latter the number of studies was limited. Toxicological information available did not allow to allocate an ADI (EFSA, 2014a).

Hazard from derivatives of propynoic acid, according to the GHS, may include harm or toxicity upon being swallowed. Ethyl 2-butynoate is classified as causing skin and serious eye irritation, and a possible respiratory irritation but no relevant information on safety regarding exposure through diet has been identified.

Nitro compounds

Despite nutritional benefits and efficacy of nitrate in reducing CH_4 emissions, variations in response often related to the feed type and its reduction to nitrite have caused concern. Nitrite absorbed in erythrocytes form methemoglobin in contact with haemoglobin, making it unable to carry oxygen. In reaction with secondary amines, nitrite can produce *N*-nitrosamines which are suspected to induce DNA mutations and cancer (IARC, 2010) and have been evaluated as raising health concerns for consumers through dietary exposure, although with many uncertainties due to limited data availability (EFSA, 2023a). Ingested nitrate or nitrite under conditions that result in endogenous nitrosation have been classified as probably carcinogenic to humans (IARC, 2017). Yet, nitrite may build-up in animal tissues following nitrate-rich diets when rates of nitrate reduction are higher than rates of nitrite reduction (Dawson, Rasmussen and Allison, 1997).

The ADIs of 3.7 and 0-0.07 mg/kg body weight per day were established by JECFA for nitrate and nitrite, respectively (FAO/WHO, 2002b). In a recent evaluation, EFSA concluded that the benchmark dose lower bound ($BMDL_{10}$) of 64 and 2 mg nitrate/kg body weight per day does not raise health concerns for ruminants and pigs, respectively (EFSA, 2020). Nitrate and nitrite supplemented in feed may leave residues in food commodities including meat and milk (Doreau *et al.*, 2018; Guyader *et al.*, 2016), although other sources such as natural presence, use as fertilizers on crops or as additives in food processing have been described to largely contribute to detected concentrations (Karwowska and Kononiuk, 2020). The use of nitrate as feed additives is banned in some countries (Beauchemin *et al.*, 2020).

As environmental pollutant linked to food safety, nitrate in excess can lead to eutrophication causing perturbations to ecosystems and has been reported to be of concern in groundwaters worldwide (Abascal *et al.*, 2022; Singh *et al.*, 2022). It is associated with harmful algal blooms (HAB) which can pose threat to human and animal health owing to the production of toxins (FAO, IOC and IAEA, 2023).

Other nitro compounds investigated as reducers of CH_4 emissions (Anderson *et al.*, 2006 and 2010; Božic *et al.*, 2009; Brown *et al.*, 2011; Gutierrez-Bañuelos *et al.*, 2007), for example nitroethane, may cause irritations when inhaled or ingested, induce the formation of methemoglobin or show toxicity at relatively low levels. However, less is known regarding their toxicity in feed supplementation (Teng and Kim, 2021). Doses high enough to efficiently reduce CH_4 emissions may be lethal to animals as it has been shown for nitropropanol (Zhang *et al.*, 2018). 3-Nitropropionate is naturally found in some fungi and plants (Parry, Nishino and Spain, 2011); its metabolization in the gut of ruminants can lead to the formation of ionized form which has been reported to irreversibly inactivate mitochondrial succinate dehydrogenase resulting in neurological disorders (Francis *et al.*, 2013). It has been associated with toxicity for grazing animals related to ingestion of plants containing 3-nitropropionate and with human poisoning upon ingestion of fungi (Su and Gadda, 2018).

Sulfur compounds

An ADI "not specified" has been allocated by JECFA for sodium sulfate due to its use in food as colour adjuvant and the absence of evidence of toxicity (FAO/WHO, 2002a). It is also an approved food additive commonly used as a filler or stabilizer, for example in chewing gum. Safety risk assessments of various sulfate-based feed additives for animal health are available.[7] Natural exposure to sulfur present in food has been considered higher than exposure to this compound used as pesticide, therefore toxicological reference values have not been considered necessary (EFSA, 2023b).

Adverse effects have been described for ruminants at high concentrations of sulfate in feed, accounting for 0.4 percent or greater of dietary sulfur (Kandylis, 1984). Polioencephalomalacia occurred in sheep fed with over 2 percent of sodium sulfate in dry matter feed, while no toxicity was reported at up to 10 g per day through continuous ruminal infusion. Concentrations of 15 g and 270 g of elemental sulfur per day in the diet of each animal resulted lethal for sheep and cattle, respectively (Kandylis, 1984). However, the negative effects of sulfur can be attenuated through various dietary and management strategies (Drenowski, Pogge and Hansen, 2014; US Grains Council, 2023). In grazing animals, possible additive effects from environmental sulfur-based contaminants must be considered. Reductions of sulfate to sulfide in rumens have been reported to rapidly build-up as toxic hydrogen sulfide (H_2S) gas having effects on the central nervous systems (Knight, 1985).

According to the GHS, hazard from sodium sulfate may involve serious eye irritation and toxicity to the gastrointestinal tract.

Ionophores

Polyether monocarboxylic acids

Concerns regarding adaptation of microorganisms to dietary ionophores due to emergence of Antimicrobial Resistance (AMR) have been raised leading to limitations in the use of these substances in animal production (Guan *et al.*, 2006; Hook, Wright and McBride, 2010; Wong, 2019). Ionophores have been deemed not to pose risk of cross-resistance to other antibiotics (FDA, 2016). However, an association with narasin and resistance to antibiotics used to treat infections in humans has been reported (VKM, 2015). In addition to a widespread presence of transferrable *narAB* gene operon conferring resistance to narasin, co-occurrence of *narAB* and clinically relevant antibiotic resistance genes has recently been detected in enterococci isolated from poultry meat (Pikkemaat *et al.*, 2022).

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[7] efsa.europa.eu/en/search?s=sulfate

Ionophore-induced toxicity, widely reviewed previously, results from charged or zwitterionic complexes they form with cations and causes perturbation of action potentials of cell membranes (Novilla, 2018). The susceptibility for toxicity effects of various animal species to ionophores may be highly variable; for example, monensin has been reported to be extremely toxic to horses (Rumbeiha and Snider, 2014).

Residues of ionophores in food have been usually reported to be far lower than safety levels. However, concentrations exceeding those values were occasionally reported for eggs and chicken tissues (VMD, 2018). Maximum Residue Limits (MRLs) exist in various jurisdictions for ionophores in tissues of livestock (Canada, 2022; Codex Alimentarius, 2021; EU, 2009 and 2017a). At the international level, the standards for MRLs are established by Codex Alimentarius for pesticides and veterinary medicines. MRLs as narasin A for cattle recommended by Codex Alimentarius are of 15 µg/kg in muscle and kidney, and 50 µg/kg in liver and fat, while the ADI was set at a maximum of 5 µg/kg body weight per day (Codex Alimentarius, 2021). For monensin, the ADI was set by Codex Alimentarius at 0-10 µg/kg body weight per day (Codex Alimentarius, 2021), with MRLs for cattle, sheep and goats of 100 µg/kg for fat, 10 µg/kg for kidney, 20 µg/kg for sheep and goat liver, 100 µg/kg for cattle liver, 10 µg/kg for muscle and 2 µg/L for milk. Cytotoxic activity of ionophores enniatins, a class of emerging mycotoxins produced by *Fusarium* spp. Fungi commonly found in temperate regions in grains and grasses, was demonstrated in several mammalian cell lines (Prosperini *et al.*, 2017). Although they have been found in trace amount in milk and other commodities of animal origin (e.g. poultry) (Křížová *et al.*, 2021; Pietruszka, Panasiuk and Jedynak, 2023), available data have not been considered sufficient to perform food safety risk assessment for enniatins (EFSA, 2014b).

Ionophores excreted by animals remain in manure and may be spread on land or to aquatic environments posing a threat to non-target organisms (Bak and Björklund, 2014; Hansen *et al.*, 2009; Žižek *et al.*, 2011 and 2015; Žižek and Zidar, 2013). More recent formulations of ionophore-containing products are considered safe for terrestrial and aquatic compartments, as well as for sediments, with low bioaccumulation potential (EFSA, 2019a).

Exposure has been reported to cause eye but not skin irritation, potential skin and respiratory sensitization, with increased risk by inhalation during handling (EFSA, 2019a).

Other compounds

Cyclodextrins

Cyclodextrins used in food and food contact materials are recognized as not posing health risks (Fenyvesi, Vikmon and Szente, 2016; Gonzalez Pereira *et al.*, 2021; Matencio *et al.*, 2020; Velázquez-Contreras *et al.*, 2022; Zhou *et al.*, 2022). The ability of cyclodextrins to inhibit methanogenesis was studied in association with other materials hosted in the hydrophobic internal cavity (Lila *et al.*, 2004; McCrabb

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Asparagopsis taxiformis in its natural environment near Australian coasts (top), its controlled culture (middle) and dehydrated kelp (bottom) as feed supplement for cows.

et al., 1997; Mohammed *et al.*, 2004a and 2004b; Rajaraman *et al.*, 2017).

According to the Generally Recognized as Safe (GRAS) status by the United States Food and Drug Administration (FDA), the application of cyclodextrins in food has been considered safe (FDA, 2000 and 2016a). The maximum advisable level of 5 mg/kg body weight per day for β-cyclodextrin used as food additive has been established by JECFA (FAO/WHO, 1995). The same value is approved by EFSA as the ADI for β-cyclodextrin (E-459), with ADI not specified for α-cyclodextrin and γ-cyclodextrin owing to their low toxicity (EFSA, 2016a; FAO/WHO, 2000 and 2005). In the EU, α-cyclodextrin and γ-cyclodextrin were approved as novel food ingredients in 2008 and 2012, respectively (EU, 2008 and 2012).

Fatty acids

Medium-chain fatty acids (MCFA) are naturally present as part of triglycerides in milk fat and feeds based on vegetable fats such as palm kernel and coconut oil (de Vrese *et al.*, 2010). Polyunsaturated fatty acids (PUFA) assessed for methanogenesis inhibition are found in plant-based sources such as soybean oil and sunflower, flax or canola seeds which are considered safe for human consumption (Adeleke and Babalola, 2020; Beauchemin *et al.*, 2009; Lills *et al.*, 2011).

While JECFA did not evaluate the entire group of fatty acids as food additives, there are values available for specific sources and processing. For oxidized soybean oil interacted with mono- and diglycerides of fatty acids approved as food additive, the ADI was first established at up to 30 mg/kg body weight per day (FAO/WHO, 1992) with further re-evaluation by EFSA based on the highest estimated exposure which indicated no safety concerns for consumers despite insufficient or missing toxicological data including genotoxicity (EFSA, 2018b). As food additives, fatty acids have been reported not to raise concerns for toxicity effects at doses up to 10 percent in the diet and not to be genotoxic, however, data on developmental, reproductive and chronic toxicity are limited (EFSA, 2017a). It has been recommended to maintain the exposure to saturated fatty acids at 1 percent on average from regular diet and food additives together (EFSA, 2017a).

Phosphonic acids

The inhibitory activity of ethephon on CH_4 emissions occurs through its metabolite ethylene. Current MRLs for ethephon established by Codex Alimentarius are of 0.01 mg/kg in animal commodities such as eggs, milk, mammalian fat and meat from mammals other than marine mammals, while values in plant-based foods range from 0.5 to 7 mg/kg (Codex Alimentarius, 2016). Upon the last evaluation by JMPR, the ADI and acute reference dose (ArfD) formerly established for ethephon were confirmed at 0-0.05 body weight per day and 0.05 mg/kg body weight, respectively (FAO/WHO, 2015), while the USEPA reference dose (RfD) for oral exposure has been set at 5 µg/kg body weight per day (USEPA, 1988). Also, human, animal and environmental safety of ethephon applied to plants as pesticide has recently been reviewed by EFSA with the recommended ADI of 20 µg/kg body weight per day (EFSA, 2023c). The main toxicity effect is considered to be associated with the inhibition of cholinesterase (ChE) activity, with dogs as the most sensitive species. It has been assessed as unlikely to be genotoxic and unlikely to pose a carcinogenicity risk to humans. Neurotoxicity due to ChE inhibition in the brain has not been considered adverse as the inhibition of ChE in erythrocytes is the most sensitive endpoint. Effects of the minor metabolite 2-hydroxyethane phosphonic acid (HEPA) were considered of minor entity, however, the need for evaluation of its aneugenic potential according to the most recent criteria has been emphasized (EFSA, 2023c). Considering incomplete data for genotoxicity, provisional risk assessment from residues in animal matrices, this evaluation indicated no risk for humans. Risk from ethephon and its metabolites to the environment and non-target organisms has been defined as low (EFSA, 2023c). MRLs for plants and animal commodities are available in several jurisdictions (Australian Government, n.d.).

When considering hazard according to the GHS classification, ethephon may be harmful when swallowed and inhaled, toxic in contact with skin causing severe burns and eye damage. It has been also reported to be toxic to aquatic organisms with long-lasting effects.

Pyromellitic diimide compounds

Several compounds with CH_4 emission inhibitory properties have been proposed (Rennison, Boddy and Brimble, 2022; Zhang and Yang, 2012), but toxicological information is extremely limited. A study in mice showed no adverse effects for (1,3,5,7-tetraoxo-5,7-dihydropyrrolo[3,4-*f*]isoindole-2,6(1*H*,3*H*)-diyl) bis(methylene) dinitrate at 300 mg/kg (Rennison, Boddy and Brimble, 2022).

Unsaturated hydrocarbons

Ethylene is present in human diet as naturally produced plant hormone which induces the ripening of fruits. It has been considered to constitute little hazard to aquatic environments and to pose no risk to human health at moderate concentrations (UNEP, n.d.). However, its metabolite ethylene oxide has been reported to have mutagenic properties, to be carcinogenic at high concentration levels by inhalation and toxic to reproduction (USEPA, 2016). In an evaluation, IARC classified ethylene oxide as carcinogenic to humans (Group 1) and considered ethylene as not classifiable as to its carcinogenicity to humans (Group 3) (IARC, 2008 and 2018a). The use of ethylene oxide in food is banned in many countries.

Acetylene is a highly flammable gas and may cause respiratory arrest when inhaled but no other adverse effects have been considered to pose a threat to human health. No ADI have been established for ethylene, ethylene oxide and acetylene by JECFA or JMPR.

2.1.2 BIOLOGICAL METHANOGENESIS INHIBITORS

The use of secondary plant metabolites able to inhibit ruminal methanogenesis has been extensively reviewed showing major efficacy *in vitro* compared to the application in livestock (Patra *et al.*, 2010). Biological methanogenesis inhibitors are usually mixtures of compounds with different percentage added to feed and, for plant-based sources, can be used as entire plant, its parts or extracts, in their natural or processed form, which must be taken into account when evaluating health risks.

MCR inhibitors and seaweed bioactive compounds

Asparagopsis seaweeds

Safety of *Asparagopsis* spp. For animals is assessed mainly in regard to bromoform, the most abundant constituent in seaweed with the ability to inhibit methanogenesis (Machado *et al.*, 2016). Other halogenated compounds, such as DBCM, are far less abundant. Food safety hazard in seaweeds and evidence of bromoform, bromide or iodine in meat and milk of ruminants fed *Asparagopsis* spp. Have recently been reviewed by FAO (FAO, 2023a; FAO 2022b).

Elevated levels of bromoform in animal products have not been detected at the minimum effective inclusion level of *Asparagopsis* spp. (Glasson *et al.*, 2022). Animal studies of up to 147 days of duration showed either metabolization or excretion of bromoform down to background levels from red seaweed supplementation in feed (Glasson *et al.*, 2022). Dehalogenation of HMAs, like bromoform and chloroform, resulting from properties of ruminant digestive tracts in combination with the action of methanogens has been suggested to limit the transfer of these substances to food products derived from ruminant livestock (Glasson *et al.*, 2022). Residues of bromoform in meat or offal have been considered undetectable (Kinley *et al.*, 2020; MLA, 2023). In other studies, concentrations of bromoform have been detected in milk from control animals and cows fed Asparagopsis at non significantly different levels, suggesting drinking water as a possible source of contamination (Roque *et al.*, 2019; Stefenoni *et al.*, 2021).

The use of bromoform-containing seaweeds in feed, similar to synthetic bromoform, needs to be thoroughly assessed considering the evaluation of bromoform by the USEPA as a probable human carcinogen (USEPA, 2005a). Moreover, signs of inflammation and abnormalities in the rumen cell wall of cows fed 67-133 g dry matter *Asparagopsis taxiformis* per day have been described (Muizelaar *et al.*, 2021).

Other seaweeds

Lower concentrations of bromoform are found in brown, green and other red seaweeds (see **Table 1**). Diet based on large amounts of seaweeds was linked to health problems in livestock from coastal areas (FAO, 2023a). The toxicity of seaweeds and possible residues of harmful substances in food commodities are variable depending on active substances specific to each species and may be related to the presence of heavy metals and minerals concentrated in tissues during physiological processes

(FAO, 2023a; FAO 2022b). Other threat may come from allergens as well as from potential bioaccumulation of biotoxins, Persistent Organic Pollutants (POPs) and other compounds such as agrochemicals or pharmaceuticals during cultivation of seaweeds in polluted areas (FAO, 2022a and 2022b, FSAI, 2020). Microbiological hazard involves a range of pathogenic bacteria and viruses and has been ascribed to contamination during post-harvest handling practices (FAO, 2022a; FSAI, 2020). The presence of marine micro- and nanoplastics on seaweed surface constitutes physical risk and can vehicle harmful chemical contaminants and bacterial pathogens from the environment to seaweed (Junaid *et al.*, 2022; FSAI, 2020). Contamination due to some of those chemicals (iodine, arsenic, metals and biotoxins) and pathogens (norovirus) is considered an emerging risk associated to seaweeds (EFSA, 2017b). Ranking of chemical and microbiological hazards from seaweeds in food and feed showed major concerns regarding the presence of arsenic, cadmium, iodine and *Salmonella*, and possible moderate hazard from lead, mercury and aluminium (FAO, 2022a). While *Bacillus* spp. And norovirus may constitute moderate hazard in food, limited data have been found for this hazard in relation to feed (FAO, 2022a).

JECFA evaluated the safety of processed *Eucheuma* seaweed and carrageenan as food additive considering data relevant to the safety assessment for infants and concluded that there is no concern of using carrageenan at concentrations up to 1000 mg/L in infant formula or formula for special medical purposes (FAO/WHO, 2014). EFSA recommended a temporary ADI of 75 mg/kg body weight per day for the same seaweed and carrageenan until data addressing current uncertainties is available and highlighted that dietary exceedance of this value is estimated as frequent and may be of safety concern for consumers (EFSA, 2018c).

Plant bioactive compounds

Essential oils

Little information is available regarding safety of essential oils in ruminant diet, their modes of action and effects on ruminal microbiota (Cobellis, Trabalza-Marinucci and Yu, 2016). Given their multicomponent nature and variability of the composition within the same plant species, which depends on factors such as changing season and geographical source, risk assessment must be performed specifically for each extract. JECFA evaluated compounds present in the garlic extract (diallyl sulfides and allyl mercaptan) and concluded that there were no safety concerns at levels of intake when used as flavouring agents (FAO/WHO, 2000). Some components of essential oils used as feed additives, for example methyleugenol or estragole in the laurel (*Laurus nobilis*) leaf oil, have been found to be genotoxic (EFSA, 2023d). Thyme (*Thymus* spp.) essential oil has been considered safe for cattle health at doses not exceeding 8 mL per day based on *in vitro* and *in vivo* studies (Silva Castro Filho *et al.*, 2021).

A range of other essential oils and their components as feed additives for all animal species has been assessed for potential health risks.[8] Residues of carvacrol, thymol, cinnamaldehyde, and diallyl disulfide supplemented at doses of 60 and 120 mg per day were not detected in milk (Hallier *et al.*, 2013).

Flavonoids

Most studies assessing the safety of flavonoids to humans and animals have been performed considering beneficial properties other than inhibition of nitrogen loss or methane emissions. Quercetin may elicit mild adverse effects at low incidence in humans (Andres *et al.*, 2017). However, no ADI has been allocated by JECFA for this compound used as food colours due to the lack of adequate toxicological data (FAO/WHO, 1978). No safety concerns from myricetin and naringin evaluated by JECFA have been indicated at levels of intake when used as flavouring agents (FAO/WHO, 2014).

In farmed animals, the use of bitter orange extract has been considered safe under 400 mg/kg for livestock and salmon, and up to 259 mg/kg for dairy cow (EFSA, 2021b). EFSA also proposed specific maximum safe concentration for other species including pig and poultry. No concerns have been identified for consumers of animal products when the same concentrations of the extract were used in feed, nor for the environment. Marigold (*Tagetes* spp.) flavonoids for use in herbal medicines did not show genotoxic effects in a battery of assays and the dose of 5000 mg/kg body weight was not associated with abnormal clinical symptoms in laboratory animals after 14 days (Wu *et al.*, 2022). Likewise, adverse clinical signs were not observed over 90 days upon repeated exposure of rats to flavonoid-rich liquorice (*Glycyrrhiza glabra*) extract at a dose of 1000 mg/kg body weight and during 14 days post-exposure to 5000 mg/kg body weight of the extract (Bhide *et al.*, 2022). Liquorice extract may present negative health effects due to the presence of glycyrrhizinic acid which may induce elevated blood pressure. High exposure was considered in regular consumers of liquorice confectionery or herbal tea containing liquorice at the intake levels exceeding 100 mg/day (FAO/WHO, 2005a). Glycyrrhizinic acid ammoniated used as flavouring agent in feed at the concentration of 0.3 mg/kg complete feed for chickens for fattening and laying hens and 1 mg/kg for the remaining animal species is considered not to increase consumers' exposure to this compound or to pose a risk to the environment (EFSA, 2015). A recent review highlighted that data regarding the potential toxicity of flavonoids to human health is still insufficient (Tang and Zhang, 2022).

Saponins

According to recent reviews, the use of saponins within recommended concentrations is considered safe for both animal and human health, although toxicity including gastrointestinal effects has been described for some active components extracted from certain plant species (Sharma *et al.*, 2023; FAO, 2023a). The toxicity and

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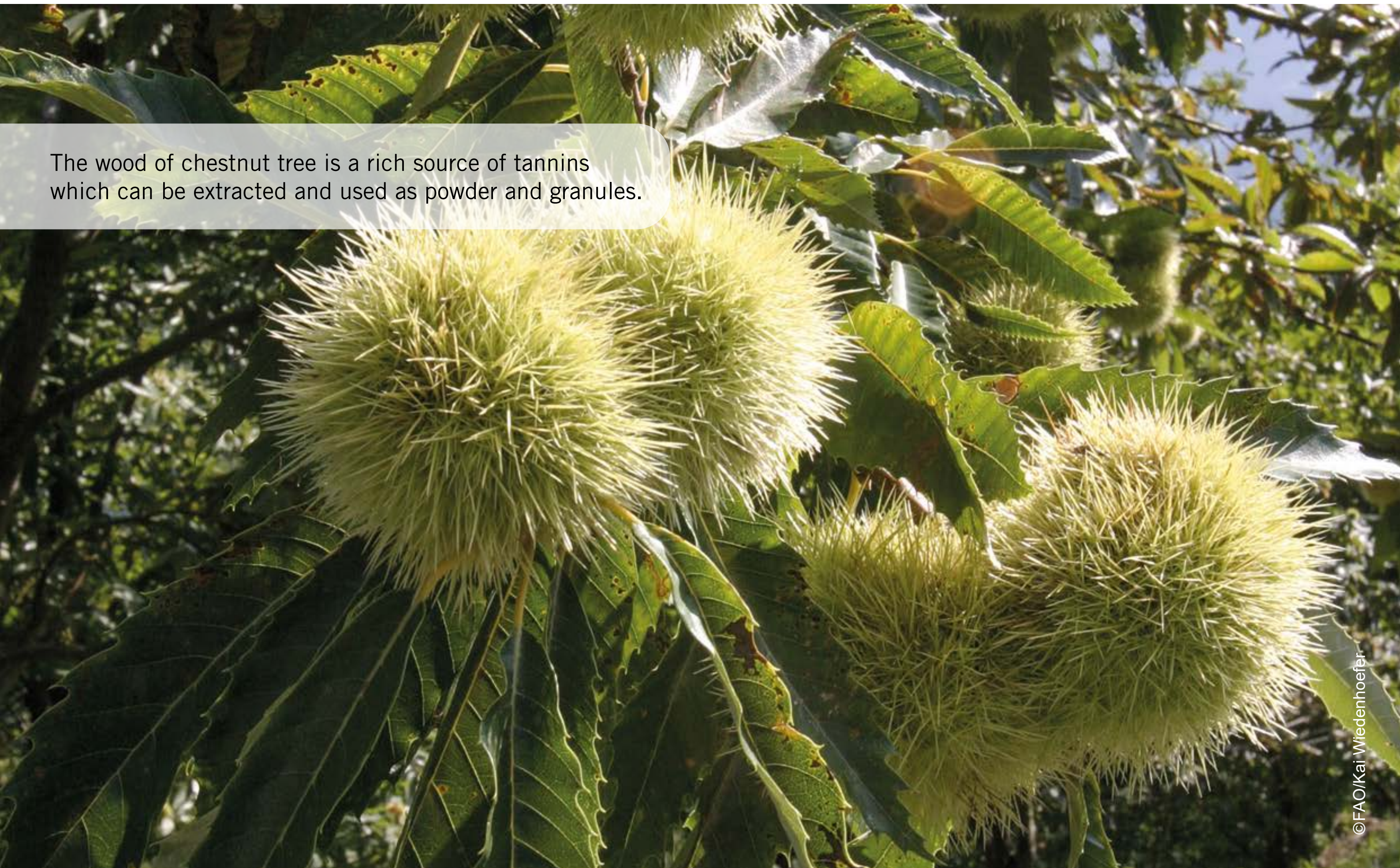
[8] www.efsa.europa.eu

safety from dietary exposure are dependent on the source and the type of saponin. The ADI for quillaia (*Quillaia saponaria*) extracts was established by JECFA at 0-1 mg/kg body weight per day (FAO/WHO, 2006 and 2014) and by EFSA at 3 mg/kg body weight per day (EFSA, 2019b). No adverse effects were observed for quinoa (*Chenopodium quinoa*) saponins in male rats below 50 mg/kg body weight and for quillaia saponins below 1200 mg/kg body weight per day with no concern for genotoxicity (EFSA, 2019b; Zhang *et al.*, 2022).

Toxicity to non-target species from quillaia saponins has been described for aquatic organisms zebrafish (*Danio rerio*) and *Daphnia magna*, and to terrestrial worm *Enchytraeus albidus*, with up to seven times milder effects caused by metabolites prosapogenin and aglycone (Adomaitis and Skujienė, 2020; Jiang *et al.*, 2018a). Toxicity of different saponin-rich plant extracts has been highlighted as a possible risk to water environments (Jiang *et al.*, 2018b).

Tannins

Potential toxic effects of tannins differ according to their classification into condensed and hydrolyzable tannins, the latter being more easily subject to microbial hydrolysis in the gut. This may result in the production of potentially toxic metabolites. However, risks to human health through food consumption has not been considered of concern (FAO, 2023a). Following safety assessments conducted on tannins as feed additives, maximum levels of use have been proposed for different livestock species at which no health concerns are expected for animals, consumers nor the environment (EFSA, 2014c and 2022a; Sharma *et al.*, 2021).



The wood of chestnut tree is a rich source of tannins which can be extracted and used as powder and granules.

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TABLE 1 EXAMPLES OF METHANOGENESIS INHIBITORS FOR REDUCING CH_4 EMISSIONS FROM LIVESTOCK RUMEN, CULTIVATED SOIL AND MANURE FOR BIOLOGICAL INHIBITORS, MAIN ACTIVE SUBSTANCES (OR THEIR CATEGORIES) ARE INDICATED ACCORDING TO THE MOST IMPORTANT PLANT SPECIES. UNCERTAIN TARGETS AND MODES OF ACTION ARE INDICATED IN GREEN

SOURCE	TYPE OF INHIBITOR	CHEMICAL CLASS AND OTHER FUNCTIONS	SUBSTANCE OR SPECIES[a]	CAS NUMBER	TARGET MICROORGANISM, MOLECULE OR PROCESS	MODE OF ACTION
SYNTHETIC						
Chemical synthesis or fermentation	Inhibitors of MCR and other enzymes in the methanogenesis pathway	Anthraquinone-based compounds (bird repellent, dyeing agent, industrial applications, human medicine)	9,10-Anthraquinone 1-Amino anthraquinone 2-Chloro anthraquinone 2-Chloro anthraquinone-3-carboxylic acid	84-65-1 82-45-1 82-44-0 84-32-2	Methanogens	Disruption of electron transfer, inhibition of ATP generation
		Chlorinated phenols (pesticides, preservatives)	2,4-Dichlorophenol 2,4,6-Trichlorophenol Pentachlorophenol	120-83-2 88-06-2 87-86-5	Enzymes in the methanogenesis pathway, acetoclastic methanogens	Non-competitive inhibition, suicide/irreversible inhibition
		Halogenated methane analogues (HMAs)	Bromochloromethane (BCM) Chloroform Bromoform Iodoform Carbon tetrachloride Bromodichoromethane 2,2,2-Trichloroacetamide Methyl fluoride Hemiacetal of chloral and starch	74-97-5 67-66-3 75-25-2 75-47-8 56-23-5 75-27-4 594-65-0 593-53-3	MCR and corrinoid enzymes, homoacetogenic bacteria	Competitive inhibition
		Nitrooxy compounds	3-Nitrooxypropanol (3-NOP)	100502-66-7	MCR	Competitive inhibition
			Ethyl-3-nitrooxy propionate 2-(hydroxymethyl)-2-(nitrooxymethyl)-1,3-propanediol N-[2-(nitrooxy)ethyl]-3-Pyridinecarboxamide Benzyl nitrate	100550-00-3 3032-55-1 65141-46-0 935-05-7	MCR, MTR	Inhibition
		Organosulfonic acids	2-Bromoethanesulfonate (BES) 3-Bromopropanesulfonate (BPS) 2-Chloroethanesulfonate (CES) 2-Mercaptoethanesulfonate (MES) (4-Hydroxyphenyl) chloromethanesulfonate (C-1)	4263-52-9 55788-44-8 15484-44-3 19767-45-4 117224-69-8	MCR	Competitive inhibition
		Pterins	Lumazine	487-21-8	Deazaflavin (F_{420})	Competitive inhibition
		Statins* (cholesterol control drugs)	Lovastatin Mevastatin	75330-75-5 73573-88-3	HCR, side effect on isprenoids in methanogenic archaea	Inhibition of isoprenoid synthesis and cell membrane formation

TABLE 1 EXAMPLES OF METHANOGENESIS INHIBITORS FOR REDUCING CH_4 EMISSIONS FROM LIVESTOCK RUMEN, CULTIVATED SOIL AND MANURE FOR BIOLOGICAL INHIBITORS, MAIN ACTIVE SUBSTANCES (OR THEIR CATEGORIES) ARE INDICATED ACCORDING TO THE MOST IMPORTANT PLANT SPECIES. UNCERTAIN TARGETS AND MODES OF ACTION ARE INDICATED IN GREEN (continued)

SOURCE	TYPE OF INHIBITOR	CHEMICAL CLASS AND OTHER FUNCTIONS	SUBSTANCE OR SPECIES[a]	CAS NUMBER	TARGET MICROORGANISM, MOLECULE OR PROCESS	MODE OF ACTION
SYNTHETIC						
Chemical synthesis or fermentation	Alternative H_2 sinks	Carboxylic acids	Propynoic acid Ethyl 2-butynoate	471-25-0 4341-76-8	Hydrogen, methanogens	Incorporation of hydrogen or formation of compounds incorporating hydrogen (e.g. malate, acrylate, crotonate)
		Nitrocompounds (food additives, industrial applications)	Nitrate Nitrite Nitroethane 2-Nitroethanol 2-Nitropropanol 3-Nitropropionate	14797-55-8 1092528-35-2 79-24-3 625-48-9 2902-96-7 504-88-1	Hydrogen, possible side effect on methanogens	Reduction to other molecules, possible inhibition of methanogens
		Sulfur compounds	Sulfate	14808-79-8	Hydrogen	Reduction to hydrogen sulfide
	Ionophores	Polyether monocarboxilic acids (anticoccidials, antibiotics)	Monensin Narasin Salinomycin	17090-79-8 55134-13-9 53003-10-4	Monovalent cations	Reversible formation of complexes with cations and transport across cell lipid membranes leading to changes in transmembrane ion gradient and electrical potential
	Other compounds	Cyclodextrins (food additives, processing and packaging, pharmaceutical and environmental applications)	β-Cyclodextrin with guest materials: Diallyl maleate Iodopropane Bromochloromethane Caprylic acid Horseradish oil	7585-39-9 999-21-3 26914-02-3 74-97-5 124-07-2	Depending on guest materials	Depending on guest materials
		Medium-chain fatty acids (MCFA) and polyunsaturated fatty acids (PUFA)*	Capric Caprylic Hexadecatrienoic Lauric Myristic Oleic Palmitic	334-48-5 124-07-2 7561-64-0 143-07-7 544-63-8 112-80-1 57-10-3	Cell membrane, methanogens	Reduction of selective permeability of cell membranes
		Phosphonic acids (plant growth regulators)	Ethephon (2-chloroethylphosphonic acid)	16672-87-0	Methanogens	Reversible inhibition
		Pyromellitic diimide compounds	Pyromellitic diimide and derivatives	2550-73-4	Methanogens	Uncertain
		Unsaturated hydrocarbons (plant growth regulators, food and industrial applications)	Ethylene Acetylene	74-85-1 74-86-2	Methanogens	Reversible inhibition

TABLE 1 **EXAMPLES OF METHANOGENESIS INHIBITORS FOR REDUCING CH_4 EMISSIONS FROM LIVESTOCK RUMEN, CULTIVATED SOIL AND MANURE FOR BIOLOGICAL INHIBITORS, MAIN ACTIVE SUBSTANCES (OR THEIR CATEGORIES) ARE INDICATED ACCORDING TO THE MOST IMPORTANT PLANT SPECIES. UNCERTAIN TARGETS AND MODES OF ACTION ARE INDICATED IN GREEN** (continued)

SOURCE	TYPE OF INHIBITOR	CHEMICAL CLASS AND OTHER FUNCTIONS	SUBSTANCE OR SPECIES[a]	CAS NUMBER	TARGET MICROORGANISM, MOLECULE OR PROCESS	MODE OF ACTION
BIOLOGICAL						
Asparagopsis seaweeds	MCR inhibitors	Organobromine compounds, HMAs	*Asparagopsis* spp. – bromoform*	75-25-2	MCR, coenzyme M methyltransferase	Competitive inhibition
Other seaweeds (brown, green and red)	MCR inhibitors, seaweed bioactive compounds	Various	*Cladophora patentiramea*, *Cystoseira trinodis*, *Dictyota bartayresii*, *Gigartina* spp., *Laminaria digitata*, *Macrocystis pyrifera*, *Padina australis*, *Pterocladia capillacea*, *Rhodymenia californica*, *Ulva* spp. – low concentrations of bromoform*, polysaccharides, proteins, peptides, bacteriocins, lipids, phlorotannins, saponins, alkaloids		Archaea, protozoa	Various based on active compounds, suppression of archaea and protozoa
Higher plants	Plant bioactive compounds	Essential oils*	Garlic (*Allium sativum*) – allicin, diallyl sulfides and allyl mercaptan) Anis (*Pimpinella anisum*), *Capsicum* spp., coriander (*Coriandrum sativum*), thyme (*Thymus* spp.), oregano (*Origanum vulgare*), mint (*Mentha* spp.), orange (*Citrus* × *sinensis*), eucalyptus (*Eucalyptus* spp.), Australian sandalwood (*Santalum spicatum*) – terpenoids, aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters, lactones	539-86-6, 870-23-5	HCR, methanogens, fermentation microorganisms. Processes associated with bacterial cell membrane	Changes in electron transport, ion gradients, protein translocation, phosphorylation, enzyme-dependent reactions
		Flavonoids	Catechin Flavone Kaempferol Myricetin Naringin Neohesperidin Poncirin Quercetin Rutin	154-23-4 525-82-6 520-18-3 529-44-2 10236-47-2 13241-33-3 14941-08-3 117-39-5 153-18-4	Methanogens, hydrogen, cell membranes	Direct inhibition of microorganisms, action as H_2 sinks via reductive dihydroxylation and cleavage of ring structures, loss of membrane integrity, inhibition of respiratory enzymes
		Saponins	Mojave yucca (*Yucca schidigera*), soap bark tree (*Quillaja saponaria*), alfalfa (*Medicago sativa*), Indian soapberry (*Sapindus mukorossi* Gaertn.), riverhemp (*Sesbania* spp.), black tea (*Camellia sinensis*)		Protozoa, methanogens, microbial fermentation, hydrogen	Inhibition of microorganisms, promotion of propionate production, incorporation of hydrogen or formation of compounds incorporating hydrogen
		Tannins*	Black wattle (*Acacia mearnsii*), sericea lespedeza (*Lespedeza cuneata*), jurema preta (*Mimosa tenuiflora*), quebracho, sweet chestnut (*Castanea sativa*), Mount Tabor oak (*Quercus ithaburensis* subsp. *Macrolepis*), common grape vine (*Vitis vinifera*)		Methanogens, protozoa, hydrogen and its production	Inhibition of microorganisms, action as alternative hydrogen sinks

*Active substances from either synthetic or biological sources.

[a] A non-exhaustive list is provided; other substances or species fall in respective chemical classes. MCR: methyl-coenzyme M reductase. HCR: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase. MTR: coenzyme M methyltransferase.

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Biological methanogenesis inhibitors can be obtained from common dietary plants such as tea (top), garlic (middle), or fennel (bottom).

2.2 NITROGEN INHIBITORS

Nitrogen inhibitors include compounds differing structurally and by modes of action. They are used to delay natural processes which lead to nitrogen loss from the soil. They act mainly at two points in the nitrogen cycle: 1) the conversion of urea into ammonia to reduce nitrogen loss through volatilization and 2) the conversion of ammonium to nitrates which are highly subject to leaching and denitrification (see **Figure 4**). The first step is targeted by urease inhibitors that specifically block the active site and reduce the activity of urease enzyme, a ubiquitous enzyme produced by soil bacteria, which mediates the hydrolysis of urea. The second step is addressed by nitrification inhibitors that slow down the activity of bacteria responsible for one or both phases of the nitrification process. Generally, nitrification inhibitors reduce the activity of enzymes responsible for nitrification, such as ammonia monooxygenase (AMO), hydroxylamine oxidoreductase (HAO) or nitric oxide reductase (NOR) (Ruser and Schulz, 2015), and eventually limit the leaching of nitrates to the soil and emissions of N_2O into the atmosphere (Byrne *et al.*, 2020). Therefore, nitrogen inhibitors delay these processes rather than prevent them from occurring, allowing time for plants and microorganisms to take up the nitrogen. Finally, denitrification inhibitors target microorganisms responsible for the transformation of nitrates into volatile nitrogen compounds, although this process has been less addressed among solutions to prevent nitrogen loss.

Nitrogen inhibitors may be applied as such or by using protected fertilizers which are treated with nitrogen inhibitors. Livestock animals can ingest nitrogen inhibitors by consuming pasture where inhibitors have been sprayed onto foliage, by directly consuming pasture or forage previously treated with nitrogen inhibitors that entered plant tissues from soil, and by ingesting soil particles with absorbed nitrogen inhibitors. Some inhibitors like dicyandiamide (DCD) have been considered for direct delivery on pasture soil in animal urine patches after being administered in feed as their recovery in the excreta of ruminants can exceed 80 percent (de Klein *et al.*, 2011; Luo *et al.*, 2015; O'Connor *et al.*, 2013).[9] Thereby, the capacity of the soil to take up inhibitors, their translocation from roots to other tissues and metabolization within plants determine the possible entry into the food chain (Adhikari *et al.*, 2019).

Limited food safety information exists regarding commodities and transformed products containing residues of nitrification, denitrification and urease inhibitors. A risk assessment model has been developed by Ray *et al.* to calculate daily exposure to nitrogen inhibitors using the example of N-(n-butyl) thiophosphoric triamide (NBPT) with risk factors and key steps along the food chain (Ray *et al.*, 2020).

The most important compounds applied as nitrification, denitrification and urease inhibitors are described below and are listed in **Table 2**.

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[9] Safety concerns are detailed in section 2.2.3.

White granules of urea fertilizer can be protected with a nitrogen inhibitor, often with the addition of colorants.

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2.2.1 SYNTHETIC UREASE INHIBITORS

Among available urease inhibitors, three substances are registered globally and commercialized under trademarks: NBPT, N-(n-propyl) thiophosphoric triamide (NPPT) and mixture NBPT+NPPT (FAS, 2023), with NBPT most widely used (Nugrahaeningtyas *et al.*, 2022).

Organophosphorus compounds

NBPT and NPPT are the most important compounds of this group, generally considered as safe both for animals and humans (Nugrahaeningtyas *et al.*, 2022). Residues of NBPT have not been found in milk and tissues of dairy cows fed with up to 10 mg NBPT/kg body weight (van de Ligt *et al.*, 2019). A study employing novel detection methods showed concentrations of NBPT and its oxidation product N-(n-butyl)phosphoric triamide (NBPTo) in milk samples below the limit of qualification (LOQ) of 0.002 mg/kg suggesting a very low residue risk of this inhibitor (Nkwonta *et al.*, 2021).

There is evidence of plants being able to take up NBPT as demonstrated for maize roots and for vegetable tissues exposed to 100 µM concentration, which inhibited urease activity in leaves and roots leading to necrosis following the accumulation of urea (Cruchaga *et al.*, 2011; Zanin *et al.*, 2015). Phytotoxicity of NBPT had a transitory nature and possibly occurred as indirect effect in areas where the inhibitor was used at high rates in combination with urea fertilizer (Edmeades, 2004). In livestock, temporary inhibition of rumen activity was observed with a subsequent adaptation of rumen microflora to NBPT, although exposure to high doses causing metabolic imbalance has been reported to be unlikely for grazing animals (Byrne *et al.*, 2020). Low concern from NBPT was shown for birds and soil invertebrates as well as for groundwater and aquatic species (NICNAS, 2011).

A five-year repeated application of NBPT coated to urea fertilizer showed no impact on the abundance or structure of soil bacterial and fungal communities of an intensively managed grassland (Duff *et al.*, 2022).

In the GHS, NBPT has been classified as causing skin irritation and serious eye damage, possibly causing respiratory irritations and suspected of reproductive toxicity. No significant hazard has been identified from NBPT levels of 0.038-0.064 percent present in fertilizer formulations, therefore it is expected that residues in food commodities may reach negligible concentrations posing low risks to consumers (NICNAS, 2011).

2.2.2 BIOLOGICAL UREASE INHIBITORS

Although a substantial number of publications exists on clinically useful plant-based urease inhibitors, relatively few studies explored the properties of analogous compounds of agricultural interest (Fernando and Roberts, 1976; Mohanty, Patra and Chhonkar, 2008; Suescun *et al.*, 2012). The identified compounds are obtained from several parts of a plant, differ in the way of application (as extract, powder or entire parts) and encompass various chemical classes.

Plant extracts, seed parts or wastes

The inhibitory activity of many extracts in this category are due to polyphenols with tannin like properties, as is the case of black tea (*Camellia sinensis*), Roman cassie (*Vachellia caven*), Monterey pine (*Pinus radiata*) or inknut (*Terminalia chebula*) extracts. Theabrownin from black tea was not associated with abnormal effects in mice after 28 days of exposure at a daily intake of up to 400 mg/kg but slight nephrotoxicity was observed upon exposure to 4 g/kg per day (Ding *et al.*, 2022). Consumption of up to 1 g/kg and 5 g/kg of black tea extract per day by humans and rats, respectively, was considered safe, although slight hepatotoxicity at the latter dose cannot be excluded (Chachiyo *et al.*, 2020; Fujita and Yamagami, 2008; Nor Qhairul Izzreen *et al.*, 2020; Wang *et al.*, 2010). Further toxicity effects and safety doses for consumption of black tea in adults have been reviewed by Hayat and colleagues (Hayat *et al.*, 2015).

Neem (*Azadirachta indica*)-based products, especially its non-aqueous extract, have been reported to cause toxicity including reversible effects on reproduction of male and female mammals elicited following sub-acute or chronic exposure as the most important endpoint. The toxicity of neem extracts has been reviewed previously (Boeke *et al.*, 2004). Azadirachtin, the active compound extracted from the neem seeds, is used as pesticide and, according to the GHS, it has been classified as skin sensitizing substance with very toxic and long-lasting effects to aquatic life. However, gaps in toxicological and safety data to consumers and non-target organisms have been confirmed for azadirachtin (EFSA, 2018d) and other plant extracts investigated as biological urease inhibitors (Li *et al.*, 2015).

Further information on toxicity endpoints of substances naturally occurring in plant extracts with possible human health concerns when present in food can be found in EFSA's Compendium of botanicals (EFSA, 2021c).

2.2.3. SYNTHETIC NITRIFICATION INHIBITORS

Numerous substances with a potential to act as nitrification inhibitors have been identified as substrates of AMO (McCarthy, 1999). Their modes of action and strategies for regulation of nitrification in agrifood systems have been reviewed by Subbarao *et al.* (2006). The chemical classes below are reported in alphabetical order.

Amide compounds

Concerns regarding DCD residues at detectable levels in buttermilk powder, whole milk powder and skim milk have been raised based on evidence from New Zealand, where DCD was applied to pastures at a rate of 10 kg/ha. Upon testing 63 samples, one raw milk sample presented quantifiable DCD level, while traces below the reporting threshold of 0.05 mg/kg were found in one raw colostrum sample (New Zealand, 2013). Subsequently, DCD has been withdrawn from the market in New Zealand even though detected concentrations are considered not to pose a risk to human health as the ADI is 1 mg/kg body weight per day, and the maximum amount of 0.5 percent as feed additive should not be of concern (EFSA, 2009; OECD, 2015). It was observed that the presence of DCD in cow milk remained at an average of 1.2 percent throughout the supplementation period and decreased to undetectable levels after 5 days when the administration ceased (Welten *et al.*, 2016). The majority of DCD was predominantly excreted in urine (82 percent), probably due to its high solubility in water and absorption in the rumen and blood. However, the authors highlighted the need to investigate the presence of DCD residues in other animal products, such as meat (Welten *et al.*, 2016). In an experiment, residues of DCD infused to sheep were detected in fat, wool, kidney and liver but not in muscle tissue (Ledgard *et al.*, 2008). In a recent study, human estimated daily intake (EDI) of DCD from milk, based on recommended application rates, according to an exposure prediction model, was below 1 mg/kg body weight per day, and therefore does not pose significant risk to human health (Ray *et al.*, 2023). The presence of DCD residues was linked to its residence time in plants, which in turn depends on the plant height and low rainfall conditions (Kim *et al.*, 2012).

It has been shown that DCD uptake and translocation to wheat shoots occurs at about 1 percent of the concentration (1 mL of 1mM solution) two hours after direct injection to the rhizosphere when in competition with root zone microorganisms (Marsden *et al.*, 2015). Based on estimates, the highest risk for acquiring DCD by grazing livestock comes from consumption of pasture that intercepted the inhibitor on their surface during spray application, however, this can be prevented by a time gap between DCD application and grazing (Marsden *et al.*, 2015).

The authors suggest that direct plant consumption may become a significant risk over longer periods due to possible DCD concentrations exceeding those in dairy products. DCD was found to affect the target organisms, i.e. ammonium oxidizing bacteria (AOB) and ammonium oxidizing archaea (AOA), but not to cause imbalance in the non-target soil microbial community (O'Callaghan *et al.*, 2010).

Toxicological information on DCD is available in various reports and databases (ECHA, n.d.a.; Matthaei *et al.,* n.d.). DCD is an active ingredient of pronitridine, also indicated as G77, which did not show safety concerns based on acute toxicity studies in animal models (NICNAS, 2020). Low environmental concentrations are not supposed to pose risk to organisms. The toxicity profile is incomplete for terrestrial invertebrates, drinking water and chronic exposure. Effects in plants have been reported to be expected only at high doses (Massachussets Department of Correction, 2019).

Pyrazole compounds

3,4-dimethylpyrazole-phosphate (DMPP) and 3,4-dimethylpyrazole-succinic acid (DMPSA) are among the main nitrification inhibitors. Their high concentrations resulted in residues accumulated in red clover plants mainly in leaves and roots, respectively, with damage from DMPP at 100 mg/kg soil (Rodrigues *et al.*, 2018). However, no phytotoxicity from both inhibitors has been observed at levels used as maximum amounts in agricultural practice (0.5 mg/kg soil). Residues of DMPP may be present following its application or due to degradation of its parent compound 3,4-dimethylpyrazole (DMP). The toxicity of DMPP has previously been reviewed by Adhikari *et al.* (2019) indicating no evidence of negative effects to soil microbes, plants and waterbodies at field application rate but with the potential to accumulate in plant aerial parts upon application and to enter the food chain following ingestion by grazing animals.

3-methylpyrazole is a metabolite of 1-carbamoyle-3-methylpyrazole (CMP) which has been reported to leave residues in plants up to 0.1 ppm taken up by roots or leaves (ECHA, 1984).

According to the GHS, hazard from pyrazole compounds may include damage to the unborn child and toxicity for reproduction. Some compounds may be toxic or very toxic to aquatic life with long-lasting effects. 3-methylpyrazole is under assessment as an endocrine disrupting chemical.

Pyridine compounds

Nitrapyrin is the main nitrification inhibitors of this chemical category. According to USEPA, there is no reasonable risk from nitrapyrin and its metabolite 6-chloropicolinic acid (6-CPA) from dietary exposure through water and food (USEPA, 2022c). Likewise, residues in meat, milk, poultry and eggs are not expected, while estimated environmental levels in groundwater and surface water have not been reported to be of concern (USEPA, 2022c). However, levels may be exceeded when nitrapyrin products are not immediately incorporated in soil upon application, posing potential risks to aquatic organisms (NZAGRC, 2019; USEPA, 2005b). This may be due to nitrapyrin mobility owing to high volatilization from soil, leaching and degradation to 6-CPA, therefore incorporation in 5-10 cm of a minimum depth has been recommended (Espín and García-Fernández, 2014). Very low concentrations of 6-CPA have been found in run-off water and no traces in groundwater. Low concentration of 6-CPA rather than nitrapyrin could be found in plant tissues.

With regards to nitrapyrin, no evidence of genotoxicity, neurotoxicity and immunotoxicity was found although further studies have been recommended for the latter two (Espín and García-Fernández, 2014). Effects on reproduction and development were observed in laboratory animals and carcinogenicity effects have been suggested based on mutagenicity as mode of action. A detailed summary on the toxicity of nitrapyrin as well as evidence of entry into the food chain via grazing animals have been provided previously (Adhikari *et al.*, 2019; Espín and García-Fernández, 2014).

JECFA evaluated 3-ethylpyridine and concluded that there was no safety concern at level of intake when it is used as a flavouring agent (FAO/WHO, 2005a). 3-Ethylpyridine and other piyridine derivatives species have been considered safe for the target animals at the proposed use of 0.3-0.5 mg/kg of complete feed for various animals and for consumers when these compounds are used up to the highest level in feed, but no conclusion was drawn regarding their safety in drinking water due to lack of data (EFSA, 2016b). The same concentrations are also evaluated as safe for aquatic and terrestrial environments (EFSA, 2016b). When used as flavouring substances in food, pyridine derivatives have been assessed as not posing safety concerns at levels of intake estimated through the "Maximized Survey-derived Daily Intake" (MSDI) approach (EFSA, 2014d). According to the GHS, the following hazards are associated with 3-ethylpyridine: skin and serious eye irritant, respiratory irritation, and it has been described to possibly cause genetic defects and cancer.

Other heterocyclic compounds

Toxicological data regarding compounds of this category are very limited when considering the specific use as nitrification inhibitors. However, important groups of pesticides are based on triazole or pyrimidine and their derivatives for which risk assessments have been performed.

For pyrimidine and pyridine compounds used as herbicides, no risk concern was raised for products assessed by the USEPA, while non-target plants can be affected through run-off or spray drift (USEPA, 2022d). Some of these compounds have been reported to persist in plant tissues and cause toxicity effects to cultivated species when applying compost into which those parts have been recycled or manure from animals grazed on treated areas. For this reason, mitigation measures have been introduced to avoid toxicity from pyrimidine/pyridine compound residues (USEPA, 2022d).

Toxicity from triazole compounds applied as pesticides includes effects on various endpoints (Fishel, 2014) and has been reviewed in the context of water pollution (Huang *et al.*, 2022). The active substances of triazole pesticide are metabolized to compounds known as triazole derivative metabolites (TDMs), residues of which have been detected in food commodities from both conventional and organic agriculture at levels above the LOQ of employed methods (Ströher Kolberg *et al.*, 2016). The ADI and ARfD for 1,2,4-triazole as a common metabolite of triazole pesticides have been established by the JMPR at 0-0.2 body weight per day and 0.3 mg/kg body weight, respectively (FAO/WHO, 2015). The evaluation of consumer exposure to TDM residues in agricultural products of plant and animal origin is affected by numerous uncertainties and requires further data, although internationally estimated daily intake (IEDI) did not exceed the ADI for single metabolites demonstrating an unlikely risk for the consumers (EFSA, 2018e).

The presence of phenylhydrazines in food commodities can occur naturally (Andersson and Gry, 2004). Toxicity effects of phenylhydrazine to humans have been extensively described, although human toxicological data were considered insufficient to characterize all risks (UNEP/ILO/WHO, 2000). Phenylhydrazine derivatives can potentially inhibit non-target microorganisms in soil, therefore the development of less toxic analogues was recommended (Wu *et al.*, 2012; Yang *et al.*, 2017).

Similar to inhibitors belonging to other chemical classes, pyrimidines have been reported to cause skin and serious eye damage and may cause respiratory irritation when inhaled. In addition, pyrrole-based compounds can be toxic or fatal when swallowed. Etridiazole is considered a skin sensitizer and probable human carcinogen (Group B2) (USEPA, 2020). 3-amino-1,2,4-triazole has been reported to be toxic for reproduction, potentially teratogenic, carcinogenic and may disrupt the production of thyroid hormones (Smith, 2011). Based on the evaluation by ECHA, phenylhydrazine was classified as carcinogenic, skin sensitizing and suspected mutagenic (ECHA, n.d.b.), while the USEPA assigned this compound to Group B2 as probable carcinogen (USEPA, n.d.c.). Although the substance is considered toxic to aquatic organisms, the environmental risk has been evaluated as low because the substance is readily biodegradable (UNEP/ILO/WHO, 2000).

Quinone and quinoline-based compounds

Ethoxyquin has been used as pesticide and food and feed additive due to its antioxidant properties. Tolerances for ethoxyquin residues in animal food commodities for human consumption have been established as MRL in various jurisdictions in the range of few mg/kg or less, while they are not tolerated in milk. An MRL established by Codex Alimentarius is only available for pears at 3 mg/kg referring to post-harvest treatment (Codex Alimentarius, 2009). Ethoxyquin has been evaluated by JMPR that set the ADI and ARfD at 0-0.005 body weight per day and 0.5 mg/kg body weight, respectively, (FAO/WHO, 2005b). Currently, ethoxyquin is scheduled for re-evaluation by JECFA (FAO/WHO, 2023).

The feed additive ethoxyquin per se is considered safe for all animal species and consumers at a maximum total concentration of 50 mg/kg complete feed (EFSA, 2022b), although 150 mg/kg is allowed by FDA (FDA, 2023). However, no safety level could be established for long-living and reproductive animals due to the presence of p-phenetidine (EFSA, 2022b). Likewise, due to lack of data on the presence of p-phenetidine in animal tissues and food products (EFSA, 2022b), it could not be concluded whether ethoxyquin poses a risk to consumers. P-phenetidine is a suspected mutagenic, carcinogenic and skin sensitizing substance, and it remains as impurity in the additive at concentrations up to 2.5 mg/kg following the manufacturing process. The use of ethoxyquin in feed is not authorized in the EU (EU, 2022b).

Concerns regarding possible genotoxic effects of ethoxyquin quinone imine (EQI), one of the main metabolites of ethoxyquin, have not been sustained based on *in vivo* studies that did not report genotoxic activity of EQI observed *in vitro* (EFSA, 2022b). When analysing soil microbiota, EQI induced changed in the composition of bacterial and fungal community, suppressing *Sphingomonas* involved in the biodegradation of environmental contaminants and plant pathogens such as Fusarium but favouring other beneficial genera (Papadopoulou *et al.,* 2020).

Sulfur compounds

Thioethers used as flavouring agents have been evaluated by JECFA concluding that there were no safety concerns at levels of intake (FAO/WHO, 2000). Sodium thiosulfate was considered not to raise safety concerns when used as food contact material with respect to established limits (EFSA, 2013). As established by JECFA, the ADI for sodium thiosulfate expressed as sulfur dioxide falls in the range of 0-0.7 mg/kg body weight per day (FAO/WHO, 1999).

The effects of carbon disulfide on human health have been widely described and include neurotoxicity upon chronic exposure and reproductive effects following inhalation with restrictions in use in different countries (ATSDR, 2014; Canada, 2000; CDC, 2019; EPA, 2016; NICNAS, 2014; NRC, 2009). However, no ADI has been allocated for this compound by JMPR (FAO/WHO, 1965).

Similar to carbon sulfide, thiourea is a substance suspected of being toxic to reproduction and carcinogenic (NTP, 2021; UNEP/ILO/WHO, 2003). It has been reported to cause long lasting effects in aquatic organisms. Thiourea and its derivatives were found to elicit toxicity effects in the thyroid gland, which at dosages able to inhibit thyroid function were associated with reproductive toxicity and possible carcinogenicity (ECHA, n.d.c.; Mertschenk *et al.,* 2013). Other substances of this group are known to cause skin and serious eye irritation and may also cause respiratory irritation.

Unsaturated hydrocarbons

Alkenes and alkynes occur widely in nature, for example ethylene in ripening fruits and vegetables, 1-octyne in lemon oil, octadecene in fish liver, butadiene in coffee, to mention just a few. Information regarding the safety of ethylene and acetylene is provided in section 2.1.1 on methanogenesis inhibitors. According to the GHS classification for hazard, other alkynes such as 1-octyne can be fatal if swallowed or upon entering airways and cause skin irritation. However, safety data related to agricultural application is limited.

2.2.4 BIOLOGICAL NITRIFICATION INHIBITORS

Root exudates remain the most studied source of biological nitrification inhibitors (BNI). They include compounds belonging to different chemical classes identified in a range of plant species across different climates.

Root exudates

Information regarding the toxicity and safety of root exudates for animals and consumers is limited. They are deemed to be an environmentally friendly alternative to synthetic nitrification inhibitors (Lu *et al.*, 2019) with minimum effects on non-target microorganisms in soil (Nawarathna *et al.*, 2021). A study investigating effects of methyl 3-(4-hydroxyphenyl) propionate (MHPP) on nitrogen fixation and emission reduction reported no toxic or side effects due to its application (Ren *et al.*, 2019). In perilla seedlings, MHPP was found to reduce primary root growth, but to markedly induce the formation of lateral roots modulating plant growth and metabolite profiles (Ma *et al.*, 2020).

The most studied root exudate is sorgoleone which shows potentially autotoxic properties dependent on sorghum (*Sorghum* spp.) variety (Coskun *et al.*, 2017; Tibugari *et al.*, 2020) and displays herbicidal effects towards weeds (Dayan *et al.*, 2010; Hussain *et al.*, 2021). As a highly hydrophobic substance, sorgoleone has been described to persist long strongly sorbed to soil particles but is metabolized by microorganisms at different rates based on several factors including its concentrations and soil pH (Dayan *et al.*, 2010; Uddin *et al.*, 2014). No adverse effects or mortality were observed in fish acute toxicity test at the nominal concentration of 1 µg/mL in water (Uddin *et al.*, 2012).

JECFA evaluated isothiocyanates and concluded that there was no safety concern at levels of intake corresponding to the use of these compounds as flavouring agents (FAO/WHO, 2006 and 2008). Allyl isothiocyanate (AITC) used as food preservative did not prove to be carcinogenic, genotoxic or to cause developmental toxicity in laboratory animal models (EFSA, 2010). An ADI of 0.2 mg/kg body weight per day was proposed considering uncertainties related to long-term and reproductive toxicity (EFSA, 2010). No safety concerns from dietary exposure are expected based on estimated levels of intake when AITC is used as flavouring agent (EFSA, 2008; FAO/WHO, 2006).

Syringic acid, based on a 14-day toxicological study in rats, was considered safe over limited time period (Mirza and Panchal, 2019). Information on food safety of zeanone was not identified; however, other naphtoquinones showed toxicity to isolated trout hepatocytes and insect larvae (Kim and Lee, 2016; Schmieder *et al.*, 2003). The toxicity of 2-hydroxy-4,7-dimethoxy-2*H*-1,4-benzoxazin-3(4*H*)-one

Substances acting as biological nitrification inhibitors are produced as root exudates by some common crops, including sorghum (*Sorghum bicolor*).

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(HDMBOA) and its metabolites has been mainly investigated in the context of a natural defence system of maize against insect herbivores (Glauser *et al.*, 2011; Zhang *et al.*, 2019). Toxicological testing of methyl ferulate and methyl p-coumarate showed no mutagenic effects and no skin irritating properties from these compounds, although slight eye irritation and haemolysis was observed at higher tested concentrations (Raza *et al.*, 2016). There is inadequate evidence on the carcinogenicity of shikimic acid classified by IARC under Group 3 (not classifiable in regard to its carcinogenicity to humans) (IARC, 2018b).

In the GHS system, many substances belonging to this category are classified as causing skin and serious eye irritations and possibly respiratory irritations.

2.2.5. SYNTHETIC DENITRIFICATION INHIBITORS

The potential of substances as denitrification inhibitors has been less explored compared to nitrification and urease inhibitors.

Pyrimidone- and triazinone-based compounds

These compounds have been identified as small molecules able to inhibit copper nitrite reductase (NirK), the key enzyme in the denitrification process performed by the target organism *Fusarium oxysporum*, a primary denitrifier in agricultural soils (Matsuoka *et al.*, 2017). The study was based on *in silico* modelling and efficacy tests, but no toxicity assessment has been performed to date.

2.2.6. BIOLOGICAL DENITRIFICATION INHIBITORS

Flavonoids

Procyanidins are the main class of biological denitrification inhibitors (BDI) falling in this category. While a large body of literature exists on their beneficial properties, more studies are needed to collect toxicological and safety data in the context of agricultural applications (Dasiman *et al.*, 2022). Procyanidin-rich extract from grape (*Vitis vinifera*) seeds administered to rats for 90 days at 2 percent of the diet showed no toxicity in rats assessed for acute and sub-chronic effects, and no mutagenic potential in three different tests (Yamakoshi *et al.*, 2002). No mutagenicity was observed in other studies. However, the dose of 2000 mg/kg was cytotoxic to mice after 48 hours post-exposure (Erexson, 2003). Some authors reported reduced numbers of micronucleated cells and chromosomal damage following exposure to procyanidine-rich extracts (Stancovic *et al.*, 2008, Sugisawa, Inoue and Umegaki, 2004). In another study, weak mutagenicity was identified in *in vivo* micronucleus test (Lluís *et al.*, 2011). Procyanidin B2 was classified as a weak skin sensitizer and not mutagenic to bacteria but it caused polyploidy in mammalian cells *in vitro*, although no structural aberrations were detected (Takahashi *et al.*, 1999). Other information on flavonoids is provided in section 2.1 on methanogenesis inhibitors.

Biological denitrification inhibitors can be extracted from a wide range of natural products including fruits, vegetables, nuts, legumes and grains.

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In the coming decades, growing food demand, including products of animal origin, will require measures to mitigate pressures of the agrifood sector on the environment.

TABLE 2 EXAMPLES OF NITROGEN INHIBITORS APPLICABLE TO CULTIVATED SOIL AND PASTURES ACCORDING TO THEIR CLASS, TARGET AND MODE OF ACTION UNCERTAIN TARGET AND MODE OF ACTION ARE INDICATED IN GREEN.

SOURCE	TYPE OF INHIBITOR	CHEMICAL CLASS AND OTHER FUNCTIONS	SUBSTANCE OR SPECIES^a	CAS NUMBER	TARGET MICROORGANISM, MOLECULE OR PROCESS	MODE OF ACTION
SYNTHETIC						

Chemical synthesis or fermentation	Urease inhibitors	Organophosphorus compounds	N-(n-butyl) thiophosphoric triamide (NBPT)
			N-(n-propyl) thiophosphoric triamide (NPPT)
			N-(n-butyl) phosphoric triamide (NBPTo)
			Phenyl phosphorodiamidate
				94317-64-3
				916809-14-8
				25316-39-6
				7450-69-3
					Urease	Competitive irreversible inhibition

	Nitrification inhibitors	Amide compounds (industrial applications)	Dicyandiamide (DCD) Pronitridine	461-58-5 1373256-33-7	AMO	Competitive/non-competitive inhibition as Cu chelator
		Pyrazole compounds (pesticides, human medicine)	3,4-Dimethylpyrazole-phosphate (DMPP) 2-(3,4-Dimethylpyrazole) succinic acid (DMPSA) 3-Methylpyrazole N-[3(5)-methyl-1*H*-pyrazol-1-yl)methyl] acetamide (MPA)	202842-98-6 EC 940-877-5 1453-58-3 EC 700-208-8	AMO	Inhibition as Cu chelator
		Pyridine compounds	2-Chloro-6-(trichloromethyl)-pyridine (nitrapyrin) 3-Ethylpyridine*	1929-82-4 536-78-7	AMO	Competitive inhibition as Cu chelator
		Other heterocyclic compounds (triazoles, pyrimidines, pyrroles, etc.)	4-Aminotriazole Etridiazole Benzotriazole 5-Methyl benzotriazole 3-Amino-1,2,4-triazole 2-Amino-4-chloro-6-methylpyrimidine 2-Cyanimino-4-hydroxy-6-methylpyrimidine N-2,5-Dichlorophenyl succinamic acid (DCS) Phenylhydrazine	30132-90-2 2593-15-9 95-14-7 136-85-6 61-82-5 5600-21-5 3977-29-5 25589-43-9 100-63-0	AMO and HAO pathways, AOA, AOB	(Irreversible) inhibition
		Quinone and quinoline-based compounds (food preservative)	6-Ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) (ethoxyquin) 2,6-Dihydro-2,2,4-trimethyl-6-quinone imine 2,4-Dimethyl-6-ethoxyquinoline (EQNL)	91-53-2 4071-18-5 612-50-0	AOB, AOA	Inhibition
		Sulfur compounds (pesticides)	Thiourea Allylthiourea Guanyl thiourea Carbon disulfide (Ammonium) thiosulfate** Thioethers	62-56-6 109-57-9 2114-02-5 75-15-0 14383-50-7, 7783-18-8	AMO	Competitive/non-competitive inhibition (as Cu chelators)
		Unsaturated hydrocarbons	Ethylene Acetylene 1-Octyne	74-85-1 74-86-2 629-05-0	AMO	Competitive inhibition, irreversible inhibition (suicidal inactivation process), production of inhibitory compounds (e.g. unsaturated epoxides)
	Denitrification inhibitors	Pyrimidone- and triazinone-based compounds	Compounds synthesized based on modelled structures		Copper nitrite reductase (NirK)	Inhibition

TABLE 2 **EXAMPLES OF NITROGEN INHIBITORS APPLICABLE TO CULTIVATED SOIL AND PASTURES ACCORDING TO THEIR CLASS, TARGET AND MODE OF ACTION UNCERTAIN TARGET AND MODE OF ACTION ARE INDICATED IN GREEN.** (continued)

SOURCE	TYPE OF INHIBITOR	CHEMICAL CLASS AND OTHER FUNCTIONS	SUBSTANCE OR SPECIES[a]	CAS NUMBER	TARGET MICROORGANISM, MOLECULE OR PROCESS	MODE OF ACTION
BIOLOGICAL						
Higher plants	Urease inhibitors	Plant extracts, seed parts or wastes	Green wattle (*Acacia decurrens*), inknut (*Terminalia chebula*), black tea (*Camellia sinensis*), neem (*Azadirachta indica*), Monterey pine (*Pinus radiata*), Roman cassie (*Vachellia caven*)		Urease	(Concentration dependent) inhibition
	Nitrification inhibitors	Root exudates*	1,9-Decanediol Methyl 3-(4-hydroxyphenyl) propionate (MHPP) Methyl-*P*-coumarate Methyl ferulate Shikimic acid Syringic acid** Sorgoleone Brachialactone 2,7-Cimethoxy-1,4-naphthoquinone (zeanone), 2-Hydroxy-4,7-Dimethoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (HDMBOA) Isothiocyanates from cruciferous plants Fusiococcanes Ophiobolanes	4470-02-4 5597-50-2 3943-97-3 2309-07-1 138-59-0 530-57-4	AMO and HAO pathways, AOB, AOA	Inhibition, loss of membrane potential and integrity
	Denitrification inhibitors	Flavonoids	Fruits, vegetables, nuts, legumes, grains – procyanidins		Membrane-bound nitrate reductase	Specific inhibition

*Active substances from either synthetic or biological sources.

**Shows also properties as urease inhibitor.

[a] A non-exhaustive list is provided; other substances or species fall in respective chemical classes. AMO: ammonia monooxygenase. HAO: hydroxylamine oxidoreductase. AOB: ammonia oxidizing bacteria. AOA: ammonia oxidizing archaea.

Increasing use of fertilizers will require measures to mitigate the loss of nitrogen from soil.

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The use of nitrogen inhibitors may have positive implications on the agrifood trade by increasing crop yield and quality while reducing fertilizer demand.

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CHAPTER 3

TRENDS IN THE USE OF ENVIRONMENTAL INHIBITORS

The continuous global population growth with the corresponding intensification of livestock farming and crop production will most likely result in the increasing trends on the application of feed supplements and nitrogen fertilizers (Alexandratos and Bruinsma, 2012; Bakken and Frostegård, 2017; van Beek *et al.*, 2010). The need for mitigation measures to face current GHG levels and emissions from agriculture on the rise as well as nitrogen loss from farmlands are likely to further advance the use of environmental inhibitors. The high price of fertilizers may hinder their use, especially in developing countries; at the same time, this may also result in the increased application of nitrogen inhibitors as practice to minimize fertilizer demand while maintaining crop yield and quality.

Several commercial products are available on the market as nitrogen inhibitors (see **Table 2**). Their use is justified especially on sites with a high input of nitrogen linked to elevated nitrogen loss (Montavalli, Nelson and Bardhan, 2013). However, the expansion of their use is hampered by inconsistent effectiveness which depends on factors such as the soil type and environmental conditions.

At present, the use of methanogenesis inhibitors is limited and mainly carried out for research purposes. Reduction of CH_4 emission may arise as a secondary effect of feed products which, in the first place, aim to enhance livestock productivity. Initiatives have been launched to collect evidence of introducing methanogenesis inhibition practices across agrifood systems in view of their possible consideration in policy development (UK, 2022). The use of 3-NOP has recently been authorized as feed additive in a range of countries worldwide including Australia, Brazil, Chile, Pakistan, Switzerland, Turkey and EU/EEA Member States (EU, 2022d) while the authorization process is ongoing for other markets such as Canada (FAO, 2023a).

Additional information on trends in use of environmental inhibitors is available in reports based on current market analysis and forecast until 2028 (Industry ARC, 2023).

Manual application of fertilizers on a traditional cultivation field is compatible with the use of nitrogen inhibitors.

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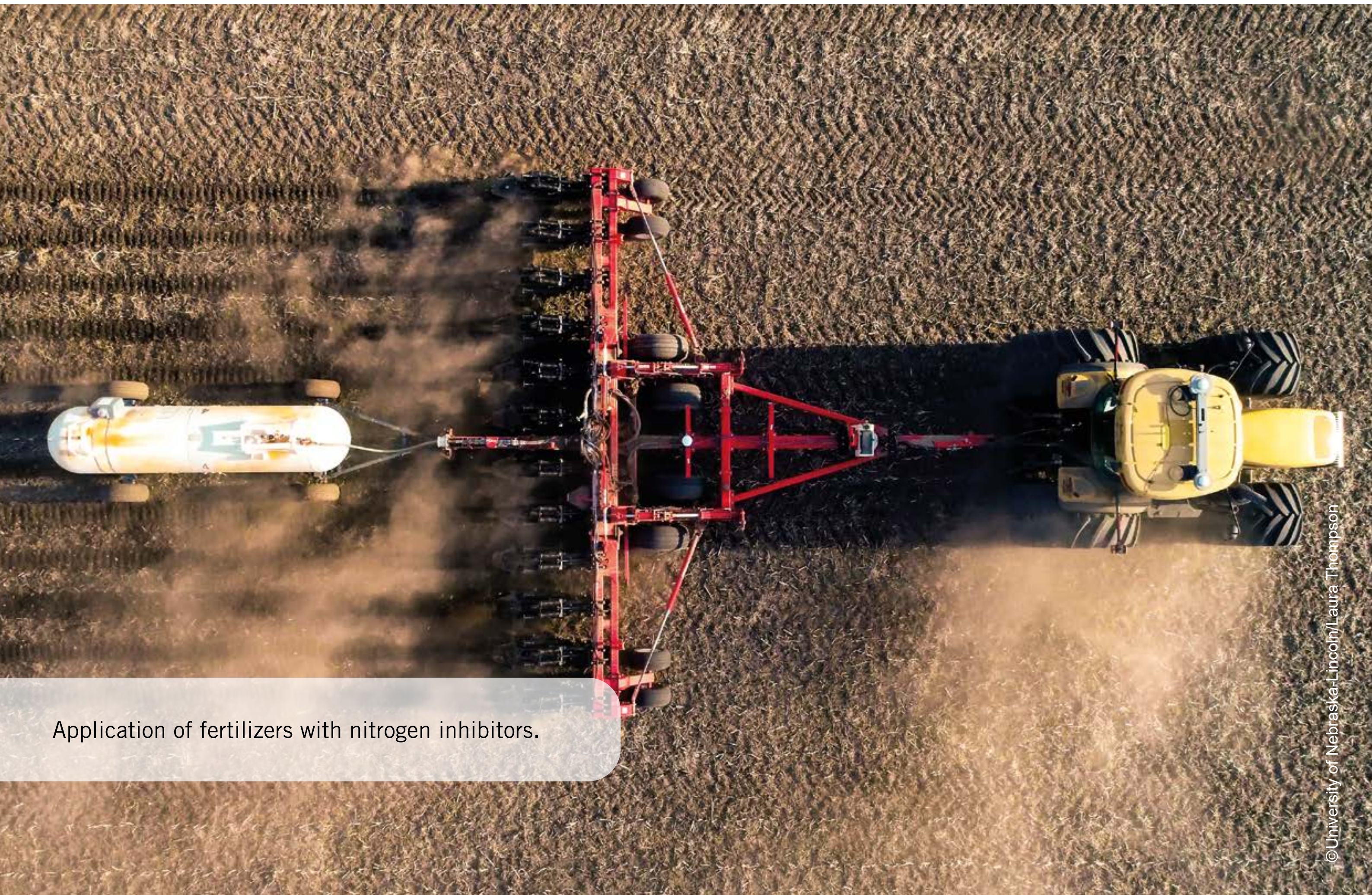

Application of fertilizers with nitrogen inhibitors.

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CHAPTER 4

REGULATORY FRAMEWORKS

While specific regulations on environmental inhibitors exist only in few jurisdictions, these compounds are often included in national and regional horizontal legislations, in particular for feed additives regulated under feed legislation, food additives and substances used in food production regulated by food safety legislation, fertilizers and pesticides. Some substances with environmental inhibitory properties are covered by legislations related to other possible uses when reduction of methane emissions or the loss of nitrogen is not the primary application (e.g. industrial use, human medicine). At the international level, Codex Alimentarius adopted guidelines for rapid risk analysis of chemical residues occasionally detected in food at low levels for which no regulatory limits exist (Codex Alimentarius, 2019). CCPR and CCRVDF both agreed that Codex MRLs for inhibitors could be considered in each committee – CCPR for crops and CCRVDF for animals (Codex Alimentarius, 2022).

This section is not intended to provide an in-depth legal analysis of regulatory frameworks related to environmental inhibitors but merely to summarize examples of regulatory approaches in different countries for which relevant information was available in the public domain and/or was provided by national authorities. As described in the following paragraphs, regional and national authorities, through available legal instruments, are undertaking efforts to ensure the appropriate food safety risk evaluation of environmental inhibitors.

ARGENTINA

In Argentina, environmental inhibitors are covered by different laws, decrees and resolutions related to agriculture and environment sectors such as the Regulatory Frameworks for Veterinary Medicines (SENASA, 2019a), the National Phytosanitary Registry (SENASA, 2019b) or the resolution on the efficacy and toxicity of phytosanitary products (SENASA, 2019c), in addition to the former Resolution on Food for Animals (SENASA, 2015) and the resolution on MRL in veterinary medicines and pesticides (SENASA, 2010 and 2011).

Under the Residue Control Plan, ionophores intended as coccidiostats together with antibiotics have been monitored in feed for several years and in 2023 more compounds of this type are going to be included. The aim is to ensure good practices in the use of ionophores in feed.

Recently, a document with Principles and Declarations of the livestock sector of the Americas was released in collaboration with other Latin American countries and USA. The document was presented at a dedicated meeting within the framework of the Regional Seminar on Sustainable Livestock Production in the Americas organized by the Argentine Rural Society (SRA), the Federation of Rural Associations of Mercosur (FARM), the Pan-American Dairy Federation (FEPALE), the Council of Dairy Exporters of United States (USDEC) and the United States Dairy Producers Federation (NMPF). The meeting proposed to strengthen the image of the livestock sector as part of the solution to global warming and the reduction of GHG.

AUSTRALIA

Australian food safety regulations apply to primary production inputs, including environmental inhibitors. As no specific risk has been identified for this class of product, their use is not currently subject to any specific regulatory provisions.

BRAZIL

Methanogenesis inhibitors are regulated as feed additives under “other zootechnical additives”, a functional group of zootechnical additives, i.e. not falling within the functional group of digestive additives, flora balancers or performance enhancers. Normative Instruction N. 1/2018 (Brazil, 2018a) included this functional group in the Normative Instruction N.13/2004 (Brazil, 2004). The technical regulation on safety of use, labelling requirements, registration and commercialization of feed additives is outlined in the Normative Instruction 13/2004, amended by the Normative Instruction N. 44/2015, with the aim to ensure an adequate level of protection of human health, animals and the environment (Brazil, 2004 and 2015).

3-NOP is currently the only product registered in Brazil with a claim of methanogenesis inhibition. Ionophores are registered in Brazil under the category of veterinary products, with the main function as growth promoters. Efficacy studies for registration purposes are currently being carried out with other potential methanogenesis inhibitors, such as additives based on tannins, natural substances that can reduce enteric methane emissions from ruminants by changing the rumen fermentation profile.

Nitrification and urease inhibitors are regulated in Brazil as authorized additives for use in mineral and organic fertilizers. According to the Normative Instruction N. 39/2018 (Brazil, 2018b), NPPT and NBPT urease inhibitors are listed for the function of reducing nitrogen loss by volatilization, with their approved use in urea. Likewise, DCD, DMPP and DMPSA are listed as substances that reduce the nitrification process in soil with approved use with urea, ammonium nitrate and nitrogen fertilizers in general. The Normative Instruction N. 39/2018 covers all aspects related to the classification, minimum levels, raw materials and additives of mineral fertilizers to be used in agriculture. The percentage of additive in the formulations is usually up to 1 percent, and values above this must be declared on the label. These same additives are listed in the Normative Instruction N. 61/2020 for authorized use in Organic Fertilizers or Biofertilizers (Brazil, 2020).

CANADA

Methanogenesis inhibitors added to livestock feed in Canada may be classified as either veterinary drugs under the *Food and Drugs Act* (Canada, 1985) or as feeds under the *Feeds Act* (Canada, 1983). The determination of the classification is dependent on the mode and location of action. For example, a product that is systemically absorbed and alters the animals' metabolism would be consistent with a veterinary drug, whereas a product that acts locally to the gastrointestinal system, could be considered a gut modifier and be registered as a feed (CFIA, 2023). The pre-market assessment of a CH_4 inhibitor as a veterinary drug or as a livestock feed would include food safety considerations consistent with their respective regulatory processes.

Nitrification and urease inhibitors are regulated by the Canadian Food Inspection Agency (CFIA) as supplements under the *Fertilizers Act* and *Fertilizers Regulations* (Canada, 2020a and 2020b), and require registration prior to import and sale in Canada. Full safety assessment with complete risk profile of the product, its mode of action and substantiation of environmental safety claims on the label must be provided (Trade memorandum T-4-127, CFIA, 2020).

CHILE

In Chile, there is no specific regulation for methanogenesis inhibitors. However, there are regulatory provisions that allow their use following authorization with objectives other than environmental inhibition. An example is a product containing 3-NOP registered and classified as a formulation additive for animal feed. The authorization of a substance for use in animal feed must comply with criteria of evaluation to verify safety and nutritional adequacy for animals and for human consumption taking into account any food product of animal origin.

Methanogenesis inhibitors are an emerging area of interest for the Ministry of Agriculture as recently discussed during the Ministerial Conference on Low Emission Food Systems[10] and also in the context of Chile's first Climate Mitigation Plan for the agricultural sector, currently in the early stages of development. Chile's national institutes are conducting research to identify GHG mitigation alternatives for the agricultural sector, commensurate with the nation's economic and productive systems.

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[10] foodsystemsmethaneconference2023.org

In accordance with Act No. 21349/2021 establishing regulations over composition, labelling and trading of fertilizers and biostimulants (formerly biofertilizers), nitrogen inhibitors are included under the definition of fertilizers (Chile, 2021). They are classified as the sixth category of products specifically acting to modify and improve fertility of the soil or its physical, chemical or biological features. Moreover, Law No. 20.412 "System of Incentives for the Environmental Sustainability of Agricultural Soils (SIRSD-S)" (Chile, 2010) supports the use of nitrification inhibitors, with the objective of minimizing loss, improving the efficiency and optimizing the use of fertilizers on crops or pastures, contributing to recovering the productive potential of agricultural soils.

CHINA

Methanogenesis inhibitors are regulated as feed additives, although no specific regulatory category has been established for them. A list of allowed feed additives published by the Ministry of Agriculture and Rural Affairs (MARA) contains those methanogenesis inhibitors which have been scientifically evaluated for efficacy and safety, registered in compliance with management regulations and approved for use and sale on the Chinese market. Among the allowed feed additives, several substances have a functional effect of reducing CH_4 emissions, such as stearic acid, sodium nitrite, sodium sulfate, saponins and some botanic extracts or mixtures (falling in the categories of "Binders, anticaking agents and stabilizers", "Preservatives, acidity regulators", "Minerals and their chelates" or "Others"). The lack of a specific category for methanogenesis inhibitors is explained by the integrated management strategy introduced to reduce CH_4 emission from livestock which combines various approaches including dietary contents. Antibiotics acting as methanogenesis inhibitors, such as the ionophore monensin, are not allowed since they are banned for use as feed additives. Currently, the introduction of 3-NOP onto the Chinese market is under evaluation.

Since 2010, Chinese authorities regulate nitrogen inhibitors, according to the fertilizer registration framework under the category of "Slow effect nitrogen fertilizers". The list of allowed substances includes NBPT, NPPT, DCD and hydroquinone.

To date, environmental inhibitors have not been separately included in Chinese Food Safety Standards for Maximum Levels of Contaminants in Foods (China, 2022a), Maximum Residue Limits for Pesticides in Foods (China, 2021 and 2022b) and Maximum Residue Levels for Veterinary Drugs in Food (China, 2019 and 2022c), due to insufficient data at national level.

EUROPEAN UNION

In the EU, methanogenesis inhibitors are regulated by Regulation (EC) 1831/2003 (EU, 2003a) on additives for use in animal nutrition, which provides information regarding authorizations and labelling requirements as well as categories of feed additives and premixtures. Article 6 refers to methanogenesis inhibitors as zootechnical additives "used to affect favourably the environment". Commission Implementing Regulation (EU) 2022/565 (EU, 2022c) authorized the use of 3-NOP as a feed additive for dairy cows and cows for reproduction on the EU market.

While ionophores used in ruminant feed generally reduce methane production in ruminants, the use of ionophore feed additives as antibiotic growth promoters in feed for ruminants has been prohibited in the EU since 2003 (EC 2003a).

The use and content of fertilizers with nitrification/denitrification and urease inhibitors in the EU falls under Regulation (EU) 2019/1009 which replaced the former Regulation (EC) 2003/2003 (EU, 2003b) in effect since 16 July 2022. Nitrogen inhibitors are considered as fertilizing products if the intention of a manufacturer is to add them to fertilizing products.[11] Regulation (EU) 2022/1519 (EU, 2022e) sets requirements applicable to fertilizing products containing inhibiting compounds including information on the product label and efficiency of fertilizers. This regulation also harmonizes the terminology relative to inhibitors, according to which "a substance or mixture which improves the nutrient release pattern of a nutrient by delaying or stopping the activity of specific groups of micro-organisms or enzyme" should be called "inhibiting compound", while the term "inhibitor" refers to the EU fertilizing products belonging to Product Function Category 5 (PFC5). The use of urea-based fertilizers coated with nitrification inhibitors is mentioned as best environmental management practice in Commission Decision (EU) 2018/813 (EU, 2018).

JAPAN

Any substance added to or mixed with the feed for the purpose of preventing deterioration of feed quality, supplementing nutritional and other active or useful ingredients or facilitating efficient utilization of nutritional ingredients is regulated by the Act for Safety Assurance and Quality Improvement of Feeds (April 1953 with the most recent amendment in June 2022) (Japan, 2022) and related ordinances, rules and notices. For a substance to be used as feed additive, it shall be designated as such by the Minister of Agriculture, Forestry and Fisheries.

Methanogenesis inhibitors are regulated as feed additives for "supplementing nutritional and other active/useful ingredients". Substances are categorized as feed additives after evaluation and confirmation of their safety to livestock, effectiveness in livestock husbandry and safety of foods of animal origin to humans.

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[11] "Substance, mixture, micro- organism or any other material, applied or intended to be applied on plants or their rhizosphere or on mushrooms or their mycosphere, or intended to constitute the rhizosphere or mycosphere, either on its own or mixed with another material, for the purpose of providing the plants or mushrooms with nutrient or improving their nutrition efficiency". Definition from Regulation 2019/1009/EU.

Their environmental effects are not evaluated. As of April 2023, there has been no feed additive designated for methanogenesis inhibition. 3-NOP has been evaluated from the point of view of safety to livestock.

Any substance applied to the ground for the purpose of plant nutrition or for changing the chemical nature of soil to facilitate plant growth, or to the plants for the purpose of plant nutrition are categorized as fertilizers according to the Act on the Quality Control of Fertilizers of May 1950 with the most recent amendment in December 2019 (Japan, 2019) and related ordinances, rules and notices. In order to manufacture a fertilizer, it shall be registered after evaluation and confirmation of its effectiveness as fertilizer. The addition of any active ingredient shall be at the least amount necessary. The "designated mixed fertilizer" can be manufactured after notification but without registration. There has been no pesticide registered in Japan that exerts functions as environmental inhibitor. No maximum levels or MRLs have been established for these substances in Japan.

Nitrification inhibitors may inadvertently decrease the quality of fertilizers containing them, therefore certain conditions for their use have been established in Japan. Only registered fertilizers not containing nitrification inhibitors and those containing one or more of the four nitrification inhibitors designated by the Minister of Agriculture, Forestry and Fisheries (1-amidino-2-thiourea, 4-amino-N-(1,3-thiazole-2-yl) benzenesulfonamide, N-(2,5-dichlorophenyl) succinamic acid and DCD) can be used alone or in mixture. After mixing, the fertilizer is categorized as "designated mixed fertilizer".

NEW ZEALAND

Environmental inhibitors are regulated by the Order 2022 (New Zealand, 2022a) of the Agricultural Compounds and Veterinary Medicines (ACVM) Act 1997 (New Zealand, 2022b) as "inhibitor substances", i.e. "active ingredients in the product that achieves the inhibitor effect", defining them as "agricultural compounds" (Ministry of Primary Industries of New Zealand, 2023). These substances are covered under the scope of the Order refers to these substances if used to mitigate adverse effects of agricultural activities on the environment, sustainability or climate change by direct or indirect management of plants and animals, application in areas with plants or animals, or added to feed or water used in animal husbandry or plant cultivation. It includes a schedule of inhibitor substances declared to be agricultural compounds along with their esters, isomers and salts, among which the most common methanogenesis, nitrification and urease inhibitors are included (e.g. 3-NOP, DCD, nitrapyrin, n-propylphosphorothioic triamide). By regulating environmental inhibitors, the Order aims to manage residues in primary produce; risks to public health, animal welfare, biosecurity and trade in primary produce; and food safety.

Environmental inhibitors for sale in New Zealand containing a substance not listed in the Order are currently not subject to ACVM. However, work is under way to amend the ACVM to include all inhibitors, which means the Order will no longer be required. Those substances listed in the Order will require registration before they can be marketed, although any substances on the market at the time the Order commenced (18 July 2023) have a two-year transitional period to obtain registration (New Zealand, 2022c). All agricultural products with an inhibitor claim will require registration to manage the risks to public health, trade, animal welfare, agricultural security, and food safety. Food Notice on MRL for agricultural compounds regulated under the Food Act 2014 (New Zealand, 2014) does not specifically establish levels for residues of environmental inhibitors in food commodities (New Zealand, 2022d).

The *Hazardous Substances and New Organisms Act 1996* (New Zealand, 1996) is administered by the New Zealand Environmental Protection Authority and was established to protect the environment and human health by preventing or managing adverse effects. It covers, among others, substances which are toxic, ecotoxic and bioaccumulative with exposure limits established for each component or element of a hazardous substance.

Products used as inhibitors that classify as hazardous substances under the HSNO Act may require a HSNO Act approval as well as registration under ACVM. Whether or not an inhibitor substance is regulated by the NZEPA, and what framework it falls under, depends on various factors including the identity and composition of the substance, concentration of the active ingredient, and method of administration to an animal or into the environment.

UNITED STATES OF AMERICA

Products that are added to animal foods may be regulated as a food or a drug, depending on whether intended effects are derived from a food attribute (taste, aroma, nutritive value) or not. Many products claiming to affect the structure or function of an animal's body currently meet the legal definition of animal drugs because they have that effect through mechanisms other than food attributes. FDA has sought public input and is re-evaluating its current policy in light of the emergence of products like some methanogenesis inhibitors and other novel animal food ingredients that achieve their intended effect solely through activity within the lumen of the digestive tract.

Federal Insecticide, Fungicide, and Rodenticide Act (USEPA, 2012) regulates the use of nitrogen inhibitors under the term "nitrogen stabilizers" intended as substances or mixtures applied with the purpose to prevent or delay the process of nitrification, denitrification, urease production or ammonia volatilization through action of soil bacteria, with some exceptions (e.g. DCD, ammonium thiosulfate).

Risk assessments based on adequate toxicological data are fundamental to ensure food safety when adopting new practices within smart agriculture, such as the application of environmental inhibitors.

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CHAPTER 5

CONCLUSIONS AND WAY FORWARD

Environmental inhibitors are just one of the approaches to mitigate CH_4 emissions and nitrogen loss in agrifood systems while progressing towards socioeconomic well-being and adequate nutrition for the growing world population. Many compounds have been known for decades and new solutions are being developed with gradually increasing interest in biological inhibitors. Despite many environmental inhibitors being considered highly efficient, their adoption in practice, especially of methanogenesis inhibitors, can be hampered by knowledge gaps, particularly regarding possible food safety implications. As the health of humans, animals and plants is interconnected and dependent on the health of the ecosystem in which they reside, approval for the use of environmental inhibitors should be performed in accordance with a One Health[12,13] approach.

SCIENCE-BASED HOLISTIC RISK ASSESSMENT AND HARMONIZED REGULATORY FRAMEWORKS ARE IMPORTANT TO ENSURE FOOD SAFETY OF ENVIRONMENTAL INHIBITORS AND FACILITATE THEIR GLOBAL TRADE

While legislations in some countries and regions include specific legal instruments under which environmental inhibitors used in agriculture are authorized, many jurisdictions allow them through regulatory frameworks which are broader in scope and do not specifically refer to such substances. Trade implications of environmental inhibitors have recently been addressed by Codex Alimentarius, highlighting the importance of having internationally accepted MRLs based on scientific assessment and an agreed definition of environmental inhibitors (Codex Alimentarius, 2022). Harmonization of regulatory frameworks at global level would not only facilitate trade, but also support correct management of food safety across globalized agrifood systems.

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[12] www.fao.org/one-health/en

[13] www.who.int/groups/one-health-high-level-expert-panel

R&D OF NOVEL ENVIRONMENTAL INHIBITORS NEEDS TO PROCEED IN CONCERT WITH RISK ASSESSMENT

Re-purposing old chemistry to the role of environmental inhibitors should be supported by a critical examination of new toxicological studies to understand limitations and changes in the data requirements. Approval of new products requires substantial efforts to evaluate their food safety implications, including modes of action, levels of possible residues in food commodities and potential harm to consumers. Thorough environmental fate studies have often been performed for products containing environmental inhibitors currently on the market and for most of the assessed substances, the residues found in food are below safety concern levels. However, food safety information is in many cases incomplete or missing. New approaches in the development of next-generation GHG inhibitory solutions, for example recombinant DNA technology for crops to increase their BNI capacity (Subbarao *et al.*, 2021), will need to be considered and evaluated. The Genetic Technology (Precision Breeding) Act that recently passed into law in the United Kingdom is opening up this area (UK, 2023).

The advancement of research for new products along with assessment of the requirements for their use, as well as the related costs implications will likely determine the selection of the best matching combinations among available mitigation approaches (FAO, 2023a; Norton and Ouyang, 2019; Patra *et al.*, 2017; Subbarao *et al.*, 2017). Challenges with field application of environmental inhibitors have already been encountered within pasture-based systems. In those scenarios, direct interactions of substances with the environment, the likelihood to impact multiple off target parameters and a lower potential for controlling the overall performance compared to stall-based systems must be considered (Dawson *et al.*, 2011). Following the withdrawal of DCD in New Zealand, ongoing research projects seek to develop novel nitrification inhibitors giving lower levels of residues in food commodities as well as novel tools and delivery methods for methanogenesis inhibitors (NZAGRC, 2023a and 2023b). Examples of near-market ready methanogenesis inhibitors have been provided for selected strategies by Searchinger *et al.* (2021).

NEW TECHNOLOGIES CAN HELP IN OPTIMIZING THE APPLICATION OF ENVIRONMENTAL INHIBITORS

Uncertainties in estimating national CH_4 and N_2O emissions in agrifood systems have been addressed by refined IPCC guidelines (IPCC, 2019). As noted in a recent FAO report, available CH_4 measurement methods involve trade-offs between ease of use, repeatability and applicability to stall-based or grazing systems (FAO, 2023a). New and/or improved detection techniques and biomarkers for CH_4 emission from enteric fermentation, manure management systems and land will allow to calculate the efficacy of inhibitors with more precision. These new techniques will also simplify the application of environmental inhibitors on commercial farms for long-term measurements (Hristov *et al.*, 2018; Tedeschi *et al.*, 2022; Thompson and Rowntree, 2020). This process will ultimately help better address the challenges posed by climate change, by providing additional GHG mitigation measures.

The development of advanced detection methods for active compounds or their metabolites in complex matrices would strengthen food safety management. However, a higher analytical sensitivity would require new regulatory approaches for dealing with higher number of detects in foods. Steps ahead to reduce labour and matrix effects are ongoing as shown, for example, by the study employing a method for detection of NBPT and its oxidation product NBPTo in milk (Nkwonta *et al.*, 2021).

Understanding changes in rumen ecosystems upon exposure to environmental inhibitors is another challenge. Progress in omics technologies can help obtain descriptive information on the complex nature and functionality of ruminal microbiome with the aim of investigating the efficiency of mitigation strategies on CH_4 formation and livestock performance.

THE ECONOMIC IMPLICATIONS OF USING ENVIRONMENTAL INHIBITORS THOROUGH CONSIDERATION OF TRADE-OFFS AND OTHER FACTORS

Agrifood policies will increasingly need to respond to the expanding demand for food as well as to transformations in agrifood systems towards smart and more sustainable agriculture. Some cost-to-benefit ratio calculations indicate that costs of inhibitor application may be outcompeted by savings when considering societal advantages related to the reduction of GHG emission for human and ecosystem health (Hu and Schmidhalter, 2021; Zhang *et al.*, 2020).

As economic return to the producer is a key driver for additional investments (Hristov *et al.*, 2013b; Subbarao *et al.*, 2017), incentives are among factors foreseen to promote the adoption of GHG mitigation strategies in practice (Herrero *et al.*, 2016). Adoption of incentives and practices for GHG abatement in the livestock sector have to reckon with trade-offs (Herrero *et al.*, 2016). Previous experiences in reducing enteric CH_4 emissions showed that investments for increasing awareness, knowledge sharing, advisory service and capacity building are required to maintain synergies with development objectives in various areas globally, so that gains in terms of GHG reduction do not negatively affect other variables (FAO/NZAGRC, 2019; Ungerfeld, 2022). Building the trust of involved parties will be crucial to integrate food safety consideration of new inhibitory substances in the very first phase of their development.

Increasing awareness among farmers, industry and consumers will favour the adoption of smart agriculture measures for a sustainable and efficient agriculture.

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FOOD SAFETY IMPLICATIONS FROM THE USE OF ENVIRONMENTAL INHIBITORS IN AGRIFOOD SYSTEMS

The challenge of feeding a growing world population while responding to the climate crisis, requires development of practices and technologies to increase sustainability of agrifood systems and reduce harmful effects on the environment. Among those approaches, environmental inhibitors are used to improve the production efficiency of crops and livestock while reducing emissions of greenhouse gases such as methane or limiting the loss of nitrogen from cultivated fields and pastures.

An inadvertent presence of environmental inhibitors in food commodities can raise health concerns as well as trade disruption if standards are not established. Challenges related to food safety risk assessment and management of these substances include the lack of internationally harmonized maximum residue limits (MRLs), agreed definition for environmental inhibitors and insufficient safety information for some compounds.

This publication provides an overview of various synthetic and biological environmental inhibitors along with an analysis of possible food safety implications from their use. Regulatory frameworks relevant for environmental inhibitors in selected countries are presented as examples of current approaches being taken at national or regional level. Finally, food safety-related knowledge gaps are discussed together with some perspectives on how to move forward.

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