Safety Assessment: Outcome of assessment of 3-Nitrooxypropanol "3-NOP" as a feed additive for all ruminants for milk production and reproduction, from DSM Nutritional Products

Reference number RP1059

Regulated Products Risk Assessment Unit

Science, Evidence and Research Division, FSA

Risk Assessment Team

Science Division, FSS

Regulated Product Dossier Assessment

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Summary

An application was submitted to the Food Standards Agency in April 2021 from DSM Nutritional Products Ltd., UK ("the applicant") for the authorisation of an additive (Bovaer® 10) containing a minimum 10% 3-nitrooxypropanol (3-NOP), under the category of 'zootechnical' additives, functional group 'substances which favourably affect the environment'.

The additive is a preparation of a minimum 10% 3-NOP, aiming to supply a minimum of 52.8 mg 3-NOP and a maximum of 88 mg 3-NOP per kg of complete feedstuff (moisture content of 12%) for all ruminants for milk production and reproduction.

To support the Food Standards Agency (FSA) and Food Standards Scotland (FSS) in evaluating the dossier, the Animal Feed and Feed Additives Joint Expert Group (AFFAJEG) and the Advisory Committee on Animal Feedingstuffs (ACAF) were asked to review the dossier and the supplementary information from the applicant. ACAF concluded that 3-NOP can be considered safe for the target species, establishing a margin of tolerance of 2. The additive can be considered safe for consumers, with an established ADI of 0.3 mg/kg bw. The additive should be considered corrosive to the eyes, a skin irritant and potentially harmful by inhalation; it is not a skin sensitizer. It was concluded the additive poses an acceptable risk to the environment. ACAF concluded that the additive can be considered efficacious.

The views of AFFAJEG and ACAF have been taken into account in the safety assessment which represents the opinion of the FSA and FSS.

1. Introduction

The FSA and FSS have undertaken a risk assessment for an additive (Bovaer® 10, DSM Nutritional Products Ltd., UK, Heanor Gate Ind. Est., Heanor, Derbyshire, DE75 7SG, UK) containing a minimum 10% 3-nitrooxypropanol, under Regulation (EC) No 1831/2003[1] under the category of 'zootechnical' additives, functional group 'substances which favourably affect the environment'. To support the safety assessment by FSA and FSS, the AFFAJEG and the ACAF provided advice to the FSA and FSS outlined in this document.

The dossier was evaluated on behalf of the FSA and FSS by the AFFAJEG. In line with Article 8 of 1831/2003, the assessment has considered whether the feed additive complies with the conditions laid down in Article 5, including: safety considerations for human, animal and environmental health; efficacy of the additive for its intended effect; potential impairment of the distinctive features of animal products This, and the guidance put in place by EFSA for the evaluation of feed additive applications, has formed the basis and structure for the assessment.

With thanks to the members of the AFFAJEG and ACAF during the course of the assessment, who were: Professor John Wallace, Professor Nicholas Jonsson, Martin Briggs, Dr. Katrina Campbell, Susan MacDonald, Professor Matthew Fisher, Christine McAlinden, Dr. Donald Morrison, Derek Renshaw, Dr. Michael Salter, Dr. Helen Warren and Dr. Nick Wheelhouse. Dr. Adam Smith declared a direct conflict of interest for the application and did not take part in the assessment.

The dossier was evaluated by the AFFAJEG at their December 2021, February 2022 and April 2022 meetings. Further information was provided by the applicant in September 2021 and March 2022, responding to queries by the Secretariat and the AFFAJEG. The conclusions by the AFFAJEG were reviewed and approved by the ACAF at their October 2022 meeting.

This document outlines the discussion and conclusions of the AFFAJEG's assessment on the safety and efficacy of 3-nitrooxypropanol as a feed additive.

2. Assessment

2.1. Section II: Identity, characterisation and conditions of use

The additive is a preparation of a minimum 10% 3-NOP (chemically synthesised), propylene glycol acting as a diluent, and precipitated and dried silicic acid acting as a carrier. The applicant provided data from eighteen batches supporting the specification values outlined below (Table 1).

able 1: Specification table

Composition	
3-nitrooxypropanol (active substance)	Minimum 10 w/w%
Silicon dioxide	~54%
Propylene glycol	~35%
Appearance	
White, free-flowing, fine granular powder	
Chemical-physical specifications	
Purity	>98.0%
Dusting potential Particle size distribution	330 – 390 mg/m^3 Average of 290 µm; 0.4% of particles with diameter < 50µm
Bulk density	0.55 kg/L

The Group evaluated the physico-chemical and technological properties of the additive, concluding that it showed good homogeneity and that it is of low dusting potential with few small particles of respirable size.

In their first evaluation, members observed that no analysis was performed on the final product to screen for dioxins and heavy metals. A question was also raised regarding the potential degradation of the product throughout the manufacturing process, as it could not be concluded whether the additive degrades in the time between production and addition to a premixture, until its incorporation in feed, and subsequently until the feed reaches the animal. The AFFAJEG requested the applicant to provide an analysis of impurities in the final product, and clarification on the potential degradation of the additive after pelleting.

The Group raised concern over the applicant's estimations of the additive's stability, as the dossier claimed that approximately 10% of 3-NOP is lost during the pelleting process. The applicant was asked to clarify whether the instruction for mash preparation would include a requirement for a higher concentration of 3-NOP to compensate for this loss. Furthermore, members estimated the average loss of 3-NOP concentration 3 months

after pelleting at 25.9%, as opposed to the 17% claimed by the applicant, and requested the applicant to revisit the stability calculations and to provide information on the process by which the additive degrades.

The applicant provided a comprehensive response addressing the Group's requests. A mistake in the stability results table was corrected, consistent with the loss of 3-NOP concentration 3 months after pelleting being 17%. It was clarified that manufacturers would be advised to use a premixture containing an additional 10% of 3-NOP in pelleted feed. The AFFAJEG estimated a percentage loss of 3-NOP (15%) during and after the production process and deemed it acceptable. No impurities were detected in the analyses presented by the applicant.

The additive is intended to supply a minimum of 52.8 mg 3-NOP and a maximum of 88 mg 3-NOP per kg of complete feedstuff (moisture content of 12%) for all ruminants for milk production and reproduction. Conditions of use of the additive are summarised in Table 2:

Table 2: Conditions of use of 3-NOP as described in the application

Proposed mode of use in animal nutrition				
Additive	3-nitrooxypropanol (3-NOP)			
CAS No	100502-66-7			
Category(-ies) of additive	Zootechnical feed additive			
Functional group(s) of additive	Substances that favourably affect the environment			
Description				
Composition, description	**Purity criteria**	**Method of analysis**		
Preparation of *3-NOP, propylene glycol and silicic acid*	Containing a minimum of: 10% w/w, 98% pure 3-NOP	HPLC system		
Trade name (if appropriate)		Bovaer 10		
Name of the holder of authorisation (if appropriate)		--		
Conditions of use				
Species or category of animal	**Min-max Age**	**Min. content**	**Max. content**	**Withdrawal period**
		mg of 3-NOP per kg of complete feed with a moisture content of 12%		
All ruminants for milk	From first insemination to culling	52.8 mg	88 mg	--

production and reproduction				

2.1.1. Conclusions on Section II

The AFFAJEG concluded that the estimated average loss of 15% of 3-NOP from production to ingestion by the animal is acceptable, particularly given the applicant's recommendation of including a 10% overage in pelleted feeds.

No further concerns were raised for Section II of the dossiers.

2.2. Section III: Safety

A set of toxicological studies and a literature review were presented and evaluated by the Chemical Risk Assessment Unit at the FSA prior to assessment by the AFFAJEG. A list of the studies contained within the application dossier can be found in **Appendix 1**.

2.2.1. Safety for the target species

The AFFAJEG evaluated two tolerance studies presented in the application.

Study 1 aimed to find a dose range to inform Study 2 and to potentially establish a margin of safety. The applicant claimed that a margin of safety of 5 could be derived from this study, but the JEG challenged this claim based on shortcomings in its design and implementation. Study 1 used only 4 cows per group, which were given 0, 100, 500 and 1000 mg 3-NOP/kg feed DM for 90 days. The highest dose showed a reduced intake of feed and a reduced heart weight, with no pathological signs (haematology, clinical chemistry, and gross pathology at necropsy and histopathology of selected organs). It was considered that such a low sample size would be unlikely to yield reliable statistics, especially given that two cows, one from the top-dose group and one from the 500 mg group, were euthanised prematurely. Furthermore, NOPA was detected in the milk from 3 out of 4 cows from the control group. The Group did not consider this study valid for evaluating the tolerance of the target species to the additive.

Study 2 used 20 cows per group, which were given doses of 3-NOP of 0, 80, 100, or 200 mg/kg feed DM for 56 days. Statistically significant differences were found in some haematological and biochemical parameters for all dose groups. These were within normal physiological ranges and without an associated dose response, therefore, were not considered to be adverse effects. At the 200 mg dose, effects identified included decreased ovary size, decreased serum activities of ALT (alanine aminotransferase) and

LDH (lactate dehydrogenase), and reduced feed and water intake. Feed and water intake vary with many factors and, since no behavioural or productivity changes (i.e., milk yield) were reported, the decrease in feed and water intake would not be considered an adverse effect. The decrease in ovarian size was not accompanied by histopathological change and it was concluded that it should not be considered an adverse effect of the study at the 200 mg/kg dose. The serum activities of LDH and ALT remained within the normal reference range and would not be considered an adverse effect. **The Group concluded that the additive could be considered safe at a dose of 200 mg/kg and that a margin of tolerance of 2 could be established.**

2.2.2. Safety for the consumer

2.2.2.1. Carcinogenicity

The applicant presented a 2-year carcinogenicity study in Wistar rats in which benign mesenchymal cell tumours were reported in 4 out of 49 females at the top dose of 300 mg/kg bw/day of 3-NOP given orally. Based on these results, the original study report concluded there was evidence of carcinogenicity in female rats. However, an independent group of pathologists reanalysed the study's slides and concluded that mesenchymal cell tumours were present in 3 out of 49 females at the top dose group, which was no longer statistically significantly different from the control group.

The AFFAJEG evaluated the data, observing that the evidence of tumour production in the medium (100 mg/kg bw/day) and low dose (50 mg/kg bw/day) female groups was inconclusive, as only one animal in each group developed mesenchymal cell tumours and this was within the historical background range of the laboratory. In addition, mesenchymal cell hyperplasia was found in two females in the top dose group (300 mg/kg/day) only. The male groups did not develop any mesenchymal tumours; however, their top dose (100 mg/kg bw/day) did produce mesenchymal cell hyperplasia. Based on this finding of mesenchymal cell hyperplasia in males at 100 mg/kg bw/day, **the NOAEL was concluded to be 50 mg/kg bw/d**.

The AFFAJEG concluded that at the higher dose levels (300 mg/kg/day in females), the additive has the potential to cause mesenchymal cell hyperplasia and benign tumours. **Due to the absence of malignant tumours and genotoxicity, it was concluded that the additive is not carcinogenic at the recommended inclusion rate and benign tumours occurred only above the NOAEL**.

2.2.2.2. Genotoxicity

The applicant presented a package of studies to evaluate the genotoxic potential of 3-NOP. The Group evaluated the positive results found in two *in vitro* micronucleus assays and an equivocal result in a third *in vitro* micronucleus assay, which contrasted with the negative findings of the two *in vivo* micronucleus studies presented. It was noted that positive results occurred in Chinese hamster V79 cells, with negative results in a study using human peripheral blood lymphocytes and an equivocal result in a study using TK6 cells. Regarding the *in vivo* negative findings, AFFAJEG experts considered that the bone marrow would have been exposed in the study using the intraperitoneal route of exposure and the negative results of this *in vivo* test should be considered valid.

In the second study, using oral dosing, the results were negative except for males dosed at the top dose and sacrificed at 24 hours, in which micronuclei were statistically significantly increased compared to the negative control, but with a frequency that was within the historical control range. Based on the OECD guidance on establishing the biological relevance of a result in this assay, which is neither clearly positive nor clearly negative, AFFAJEG members recognised the requirement for external expert judgement. An external consultant, contracted by the applicant, concluded that the apparent increase in micronuclei may have been an artifact due to the Giemsa-based stain that was used, to which Group experts agreed. **The Group concluded that 3-NOP is non-genotoxic *in vivo***.

The JEG also evaluated the genotoxic potential of 3-NOP's metabolite 3-nitrooxy-propionic-acid (NOPA). A positive result was obtained in a bacterial reverse mutation assay, but no positive results were found in a mammalian cell *in vitro* micronucleus test and an *in vivo* gene mutation and micronucleus study in transgenic mice. **The Group concluded that the metabolite NOPA is non-genotoxic *in vivo*.**

2.2.2.3. ADME

The AFFAJEG evaluated the ADME data presented by the applicant. Discussions focused on the modification of the proposed ADI, the formation of 3-NOP metabolites in the rumen and the presence of NOPA in milk and edible tissues.

The Group noted that the acceptable daily intake (ADI) proposed (discussed below) was based on toxicological data for 3-NOP and evaluated whether this ADI could also be applied to its metabolite NOPA. The application presented ADME studies performed in

rats, which showed that NOPA is the primary metabolite of 3-NOP, and ADME studies in ruminants, which demonstrated that 3-NOP is rapidly metabolised to NOPA. Levels of NOPA in the plasma of cattle also decline quickly over a period of three hours. **The Group concluded, that, given the extent of metabolism of 3-NOP to NOPA in rats, an ADI established based on toxicological data for 3-NOP could also be applied to its metabolite NOPA.**

Oxetane, a potential alkylating agent, was identified as a metabolite of 3-NOP in an *in vitro* study to investigate the metabolism of 3-NOP in goat, sheep and cow rumen fluids in the presence of feed under anaerobic conditions. However, the AFFAJEG noted that oxetane was not found in the *in vivo* studies and that it could be an artefact present in the *in vitro* study, rather than a metabolite of 3-NOP. **AFFAJEG experts noted that oxetane was very unlikely to persist within the rumen**, as it would be metabolised rapidly, with minimal release to the small intestine and negligible impact on the rumen.

From *in vitro* studies using rumen fluids, the main metabolite of 3-NOP in the rumen is 1,3 – propanediol, which is not expected to accumulate. **The Group concluded that propanediol would not be a cause for concern in the target species**. Members also noted that, in plasma, the main metabolite of 3-NOP was 3-nitrooxypropionic acid (NOPA), with other metabolites also subsequently formed, such as 3-hydroxypropionic acid within the first 24 hours.

2.2.2.4. ADI

The Group evaluated the modification by the applicant of the proposed acceptable daily intake (ADI) of 3-NOP, from 1 to 0.3 mg/kg bw/d, based on the recommendation of the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment in May 2019. The COT had been asked to advise on male reproductive toxicity observed in the chronic oral toxicity study and shorter-term studies in rats. Due to the severity of effects on the male reproductive system and the steepness of the dose-response relationship, the COT advised that an uncertainty factor of 300 should be applied, rather than the standard 100, and that the relevant point of departure for the male reproductive effects was the $BMDL_5$ of 95.6 mg/kg bw/day for decreased testicular weight. This proposed ADI also provided a margin of exposure of 167 to the NOAEL for mesenchymal cell hyperplasia in males observed in the carcinogenicity study. **The AFFAJEG concluded that for 3-NOP an ADI of 0.3 mg/kg bw can be considered appropriate**.

2.2.2.5. Exposure assessment and risk characterisation

The JEG evaluated the residue data presented in the application to determine the presence of 3-NOP and NOPA in milk and edible tissues. The applicant evaluated the exposure to NOPA from milk using the lower limit of quantification (LLOQ) level of 5 µg/kg used in the tolerance and efficacy studies presented, which showed no detectable concentrations of NOPA from all milk sampled. Toxicologist experts of the JEG carried out a conservative exposure assessment based on the JECFA food basket approach, showing that the estimate of exposure to NOPA at the LLOQ was lower than the ADI of 0.3 mg/kg bw/d established for 3-NOP by two orders of magnitude. The AFFAJEG took account of the results of all of the toxicological studies listed in Appendix 1 in deciding on the value for the ADI for 3-NOP. **The AFFAJEG compared their exposure assessment results to those presented by the applicant and concluded that, based upon the LLOQ concentration of 5 µg/kg, the levels of NOPA residues in milk were low enough not to be cause for concern.**

2.2.3. Safety for the user

The applicant presented a comprehensive set of data to support evidence of safety for the user. It was noted that the studies were carried out using the active substance (3-NOP) itself, as opposed to the final formulated product but this was not considered to be a cause for concern and tests were considered representative of the product. The studies included in the application were:

- Acute inhalation toxicity
- Local lymph node assay for skin sensitisation
- Bovine corneal opacity and permeability test for eye irritancy
- *In vitro* skin corrosion and irritation tests

Based on the data presented, the AFFAJEG concluded that the additive should be considered corrosive to the eyes and a skin irritant but not corrosive to skin or a sensitiser to skin. The applicant claimed that the additive should not be considered as harmful by inhalation, but AFFAJEG experts noted that some adverse effects were found in the acute inhalation study presented and recommended that measures to control exposure, such as masks, may need to be considered when handling the additive.

2.2.4. Safety for the environment

The AFFAJEG evaluated the environmental risk assessment carried out by the independent expert Dr. Chris Sinclair, a member of the Register of Specialists of the FSA. A detailed assessment was carried out for Phases I and II-A. The environmental risk assessment provided by the applicant was noted to have significant deviations from the expected approach, some through error but mainly due to the specific properties and behaviour of 3-NOP. However, the environmental risk assessment studies that were provided were deemed acceptable to evaluate the safety for the environment of the additive.

The environmental risk assessment evaluation of 3-NOP could stop at question 3 of the Phase I risk assessment as set out in the relevant guidance[3], since there is clear evidence that in dairy cows it is extensively metabolised to a range of endogenous compounds resulting in minimal environmental exposure. **No further assessment of the environmental risks of 3-NOP was considered necessary and it was concluded that the use of 3-NOP poses an acceptable risk to the environment**.

2.2.5. Conclusions on safety

- The AFFAJEG concluded that the additive can be considered safe for the target species at a maximum dose of 200 mg/kg DM (176 mg/kg in complete feed), establishing a margin of tolerance of 2 from the intended concentration of use.
- An ADI of 0.3 mg/kg bw/d was established for 3-NOP and NOPA.
- Metabolism of 3-NOP produces 1,3 - propanediol in the rumen. Propanediol does not accumulate in the rumen and is no cause for concern.
- Levels of 3-NOP or its primary metabolite NOPA in milk and edible tissues were not deemed as being of concern as consumer intakes would be well within the ADI.
- The additive should be considered corrosive to the eyes, a skin irritant and potentially harmful by inhalation.
- The additive poses an acceptable risk to the environment.

2.3. Section IV: Efficacy

The Group evaluated Section IV of the dossier, containing evidence of efficacy, presented in three distinct sections: three *in vitro* studies, two meta-analyses and three

long-term dairy cow efficacy trials. The rapporteurs presented the information to the group.

2.3.1. In-vitro studies

In-vitro study 1 was a straight dose-response study using doses of 0, 5, 10 and 20 mg/day of 3-NOP, showing no significant differences in response. In-vitro study 2 used a dose of 2 mg/day of 3-NOP. In-vitro study 3, used a dose of 500 mg/kg of DM, which was later adjusted to the recommended dose proposed in the application.

The Group noted that in all studies, regardless of the inclusion level of 3-NOP, methane production was significantly reduced. Members also discussed shortcomings in the in-vitro studies. Study 1 included a level of crude protein of 10.5%, which is not representative of diets for dairy cows. Study 3 presented a very high level of crude protein (23.6%), not very representative of a typical UK cattle diet and different from the control diet (17.9%). It was also noted that none of the studies used a grass, or grass silage-based, diet representative of a large proportion of dairy diets in the UK. A ruminal volatile fatty acid (VFA) reduction was observed in studies 1 and 3, as well as an increase in hydrogen levels in all three studies. The authors noted that excess hydrogen would be eructed, contributing to an energy loss. Results from studies 1, 2 and 3 can be found in Table 3, 4 and 5, respectively:

Table 3: Study 1. Effect of 3-NOP on the gas production in vitro

Gas production	Treatment (3-NOP mg)				P-value		
	0	5	10	20	Trt	Lin	Quad
Total (L / d)	1.12	1.07	1.14	1.14	0.71	0.56	0.8
CH_4 (mL / d)	27.8[a]	6.7[b]	4.3[b]	4.0[b]	<0.01	<0.01	<0.01
CH_4 (mL / g DM)	2.82[a]	0.7[b]	0.44[b]	0.39[b]	<0.01	<0.01	<0.01
CH_4 (mL / g DMD)	4.93[a]	1.25[b]	0.78[b]	0.69[b]	<0.01	<0.01	<0.01
H_2 (mL / d)	13.1[a]	33.9[b]	40.0[b]	41.6[b]	<0.01	<0.01	<0.01
[a], [b]: within a row, means without a common letter differ significantly. Trt = Treatment effect; Lin = linear effect; Quad = quadratic effect							

Table 4: Study 2. Effect of 3-NOP on the gas production in vitro

Gas production	Treatment period			Recovery period		
	Control	3-NOP (2 mg)	P-value	Control	3-NOP (2 mg)	P-value
Total (L / d)	1,27	1,10	0,03	1,14	0,98	0,06
CH_4 (mL / d)	36,5	10,4	<0,01	26,1	15,2	<0,01
CH_4 (mL / g OMD)	7,79	2,32	<0,01	-	-	-

H_2 (mL / d)	22,9	38,1	<0,01	13,1	16,2	0,53
CH_4 (%)	2,85	0,98	<0,01	2,61	1,15	<0,01
OMD: Organic matter digested						

Table 5: Study 3. Effect of 3-NOP on the gas production in vitro

Measurement	Substrate		SEM	P-value [A]
	Control	3-NOP		
Gas production (L / d) [B]				
Total	0.74	0.63	0.066	0.083
Total GHG ($CO_{2\text{-eq}}$) [C]	1.00a	0.34b	0.122	<0.001
GHG (% Total) [B, D]				
Methane	17.1a	5.0c	0.84	<0.001
Hydrogen	2.0c	10.3a	0.99	<0.001
Carbon dioxide	80.9c	84.7b	1.19	<0.001
Nitrous oxide	0.00b	0.00b	0.006	-
dH_2 [E]	40.8b	53.7a	3.79	<0.001

[A]: within a row, means with different superscripts differ ($p<0.05$)
[B]: Average of data collected in all vessels during 6 consecutive days (day 8-13)
[C]: sum of CH_4, H_2, CO_2, and N_2O produced corrected for their 100-year global warming potential (GWP) (CO_2: 1, CH_4: 28, N_2O: 265, H_2: 5.6
[D]: Gas percentages are based on the sum of CH_4, H_2, CO_2 and N_2O produced
[E]: Average of data collected in 4 vessels/day during 4 consecutive days (day 8-11)

2.3.2. Short term efficacy studies

Two meta-analyses were presented to account for short-term efficacy studies.

The first meta-analysis used twelve *in vivo* studies from ten scientific publications covering dairy cows, beef cattle and sheep, with 3-NOP doses ranging from 0 to 180 mg/kg DM. The AFFAJEG judged that the first meta-analysis should not be considered for the evaluation of efficacy, given the insufficient statistical detail presented.

The second meta-analysis evaluated data from 11 experiments and 38 treatments from 9 different studies performed in beef and dairy cattle. Members noted the positive correlation between the dose of 3-NOP and the reduction of methane. However, a negative correlation was detected with dietary neutral detergent fibre content, where the higher the NDF level, the lower the effect of 3-NOP observed in methane reduction. The Group highlighted that for 3-NOP to reduce methane concentration, it would have to be fed daily to the target animals. A summary of the data identified in the second meta-analysis can be found in Table 6:

Table 6: Meta-analysis 2. Descriptive statistics of feed intake, dietary characteristics, and CH4 emission

	Dairy Cattle				
	Mean	**Median**	**SD**	**Minimum**	**Maximum**
DMI (kg / d)	22.3	19.35	4.13	18.3	28.0
Roughage (% of DM)	55	60	7.7	38	61
NDF (g / kg DM)	319	309	52.2	265	398
CP (g / kg DM)	178	182	15.3	161	196
BW (kg)	632	664	44	573	673
3-NOP dose (mg / kg DM)	81	68	41.2	27	135
CH_4 (g / d)	351	368	94.1	132	487
MD CH_4 (g / d)	- 126	- 147	64.7	- 240	- 27
Relative MD CH_4 (% control)	- 29.6	- 30.8	16.89	- 64.5	- 6.4
CH_4 yield (g / kg DMI)	16.1	16.3	4.61	7.2	22.4
MD CH_4 yield (g / kg DMI)	- 5.2	- 5.0	2.94	- 10.6	- 1.0
Relative MD CH_4 yield (% control)	- 28.1	- 29.1	16.41	- 59.6	- 4.8
MD (mean difference) is 3-NOP group mean – control group mean					

2.3.3. Long term efficacy studies

Three long-term studies were evaluated by the AFFAJEG. The three studies shared a similar experimental design and were carried out over 19 weeks, with a target dose of 60 mg 3-NOP/ kg DM in the partial mixed ration for Holstein Friesian dairy cattle. Study 1 used 64 animals, study 2 used 42 and study 3 used 48 animals. Treatment and control groups were distributed evenly. The JEG concluded that the three studies showed similar results, with an effective reduction of methane of 21% to 33%, and that they were carried out to a high standard, with sufficient sample size and a study design that treated and fed all animals individually, where each animal can be considered an experimental unit. A summary of the results from the three long-term efficacy trials can be found in Table 7:

Table 7: CH_4 emission (g/d) of treatment groups in the three studies

	Study 1				**Study 2**				**Study 3**			
	No. cows	**Mean**	**StD**	**P-value**	**No. cows**	**Mean**	**StD**	**P-value**	**No. cows**	**Mean**	**StD**	**P-value**
Covariate period												

Control	31	464.40	54.204	-	20	406.08	48.999	-	24	368.94	43.624	-
3-NOP	32	480.43	56.110	-	20	400.19	53.013	-	24	402.61	63.202	-
Trial period												
Week 1 - 3												
Control	31	433.76	42.170		20	442.60	55.894		24	388.65	45.873	
3-NOP	32	356.87	41.303	****	20	266.50	38.421	****	24	296.73	40.706	****
Week 4 – 6												
Control	31	415.23	38.752		20	445.45	46.903		24	404.50	50.813	
3-NOP	32	340.89	41.450	****	20	287.78	34.160	****	24	312.70	46.544	****
Week 7 – 9												
Control	31	433.79	42.255		20	445.17	43.648		23	414.22	41.111	
3-NOP	32	339.18	40.595	****	20	316.82	29.948	****	24	325.66	43.684	****
Week 10 – 12												
Control	27	413.22	40.196		20	460.93	43.179		23	389.06	43.134	
3-NOP	31	332.93	44.549	****	20	285.35	33.814	****	24	319.93	54.181	****
Week 13 – 15												
Control	27	400.18	40.859		19	456.91	37.122		23	412.86	46.368	
3-NOP	31	313.06	40.338	****	20	297.02	30.259	****	24	328.13	43.807	****
Total Period												
	Nb cows	**LS Mean**	**StdE**		**Nb cows**	**LS Mean**	**StdE**		**Nb cows**	**LS Mean**	**StdE**	
Control	31	423.24	4.375		20	449.13	5.741		24	410.35	5.696	
3-NOP	32	331.62	4.297	****	20	292.05	5.735	****	24	308.11	5.639	****
Diff. (%)	- 21.6				- 35				- 24.9			
StD: Standard Deviation / StE: Standard Error / ****: p<0.0001												

The AFFAJEG concluded that 3-NOP is efficacious at reducing methane excretion in ruminants at the proposed dose. No negative effect in animal production was observed.

2.3.4. Conclusions on efficacy

The AFFAJEG concluded that the product can be considered efficacious for reducing methane production in ruminants when used on a daily basis at the proposed dose.

The AFFAJEG noted that a theoretically predicted increase in ruminal propionate and ruminal energy efficiency arising from excess hydrogen was not demonstrated.

3. Analytical methods evaluation

Conclusions on the analytical methods are presented here as an extract from the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of the Analysis for Bovaer® 10^4:

“In the current application authorisation is sought under Article 4(1) for 3-nitrooxypropanol (preparation of minimum of 10% of 3-nitrooxypropanol) under the category/functional group 4(c) ‘zootechnical additives’/ ‘substances which favourably affect the environment’, according to Annex I of Regulation (EC) No 1831/2003. Specifically, the authorisation is sought for the use of the feed additive for dairy cows and cows for reproduction, dairy sheep and ewes for reproduction, dairy goats and goats for reproduction, other ruminants for milk production and reproduction.”

For the quantification of the 3-nitrooxypropanol content in the feed additive, premixtures and feedingstuffs the applicant proposed a single-laboratory validated method based on reversed phase high performance liquid chromatography (HPLC) coupled to spectrophotometric (UV) detection.

The following performance characteristics were reported by the applicant in the frame of the validation studies for the quantification of 3-nitrooxypropanol content:

- In the feed additive: a relative standard deviation for repeatability (RSDr) ranging from 0.2% to 1.0%; a relative standard deviation for intermediate precision (RSDip) ranging from 0.3% to 1.0%; and a recovery rate (Rrec) ranging from 100% to 101%.
- In premixtures (2,870–17,390 mg/kg): a RSDr ranging from 0.4% to 1.1%; a RSDip ranging from 0.8% to 1.5%; and a Rrec ranging from 100% to 101%.
- In feedingstuffs (29–132 mg/kg): a RSDr ranging from 0.6% to 5.2%; a RSDip ranging from 1.0% to 5.2%; a Rrec ranging from 98% to 101%; and a limit of quantification (LOQ) ranging from 8 to 14 mg of 3-nitrooxypropanol/kg feedingstuffs.

Based on the experimental evidence available the EURL recommends for the official control the above mentioned single-laboratory validated and further verified reversed phase HPLC-UV method for the quantification of 3-nitrooxypropanol in the feed additive, premixtures and feedingstuffs.

FSA/FSS accepts the EURL analytical method evaluation reports. FSA/FSS determined the analytical method as appropriate for official controls for this feed additive.

4. Conclusions

The additive was fully characterised in the application and no causes for concern were identified by the AFFAJEG in the identity and characterisation.

The AFFAJEG concluded that the additive can be considered safe for the target species at a maximum dose of 200 mg/kg DM (176 mg/kg in complete feed), establishing a margin of tolerance of 2. An ADI of 0.3 mg/kg bw was established. Metabolism of 3-NOP produces 1,3 - propanediol in the rumen. Propanediol does not accumulate in the rumen and is no cause for concern. Concentrations of 3-NOP and its metabolites in milk and edible tissues are not expected to reach levels of concern. The additive should be considered corrosive to the eyes, a skin irritant and potentially harmful by inhalation; it is not a skin sensitizer. It was concluded the additive poses an acceptable risk to the environment.

Based on data from in-vitro studies, two meta-analysis and three long-term efficacy trials, the AFFAJEG concluded that the product can be considered efficacious for reducing methane production in ruminants when fed daily at the proposed dose of 52.8 - 88 mg/kg of complete feed.

5. References

1. EC (European Commission), 2003. Regulation No 1831/2993 of the European Parliament and of the Council on additives for use in animal nutrition. Available at https://www.legislation.gov.uk/eur/2003/1831/contents
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3. EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). Guidance on the assessment of the safety of feed additives for the environment. EFSA Journal 2019;17(4):5648, 78 pp. https://doi.org/10.2903/j.efsa.2019.5648
4. EURL-FA (European Reference Laboratory for Feed Additives), 2020. Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003. 3-Nitrooxypropanol. Available at: https://joint-research-centre.ec.europa.eu/publications/fad-2019-0057_en#details

6. Appendix 1: List of toxicological studies

Table 8: Studies presented in support of Section III: Safety for 3-NOP.

Study	Year	OECD	Animals	Doses tested
Tolerance and residue studies				
Pilot tolerance study, 90 days	2018	n/a	16 (4 x 4 groups) dairy cows	0, 1.6, 8, 16 g 3-NOP/cow/day = 100, 500 and 1000 mg/Kg feed DM
Pivotal tolerance study, 56 days	2019	n/a	80 (20 x 4 groups) dairy cows	0, 80 ,100, 200 mg 3-NOP/Kg DM
Milk analysis for NOPA from University of Reading efficacy study	2019	n/a	5 dairy cows	Milk samples from 5 cows receiving 3-NOP @ approx. 60 mg/kg, during 3 days in week 1, 6 and 15
ADME				
Stability of 3-NOP under Different Conditions	2015	n/a	n/a	202 µmol/L
Stability of 3-NOP under Different Conditions II – Plasma Protein Binding and Chemical Oxidation	2017	n/a	Wistar rat plasma	34 µmol/L
Plasma Protein Binding of 14C-NOPA	2019	n/a	Wistar rat plasma	31.3 µmol/l and 6.26 µmol/l at 37°C for up to 24 h
Stability of 3-NOP under Different Conditions III – In-vitro Incubations Leading to the Major Metabolite NOPA	2017	n/a	Rat (Wistar & Sprague-Dawley), Dog (Beagle) and Human Liver Fraction	34 - 36 µmol/L
Metabolite Profiles and Kinetics of 3-NOP after In-vitro Incubation	2014	n/a	Cow rumen fluid	2.2 and 23 mg/L at 38°C for 24 h
Metabolite Profiles of 3-NOP after In-vitro Incubation	2016	n/a	Sheep, Goat and Cow Rumen Fluid	1 mg/L at 39°C for 16 h
ADME tissue distribution and plasma kinetics	2013	417	Wistar rats	505 mg/kg bw
ADME in the Rat Following Single and Multiple	2018	n/a	4M/4F Wistar rats	2 exps each with 50 and 500 mg/kg bw (4 exps in total). 50 given as a single dose and as a 50 x 5 daily doses. 500 just as single doses

Study	Year	OECD	Animals	Doses tested
Oral Administration				
ADE with volatiles	2015	417	Wistar rats	506 mg/kg bw
Metabolites in plasma, liver and GIT	2014	417	Wistar rats	505 mg/kg bw
Nitrate/ nitrite in plasma	2014	417	Wistar rats	100 and 500 mg/kg bw
3-NOP in lactating goats	2015	503	2 goats	7 daily doses of 4.34 and 3.28 mg/kg bw being equiv to 112, 102 mg / kg DM (feed)
ADME in Dairy Cattle Following Multiple Oral Administration	2018	n/a	4 x Dairy cows	Every 12 hours for 7 days at dose level of 3.6 mg / kg bw / d (1.8 g / animal / d) being equiv to 150-160 mg / kg DM (feed)
ADME in Dairy Cattle Following Multiple Oral Administration (part 2)	2021	n/a	10 x Dairy cows	Every 12 hours for 5 days at dose level of 3.6 mg / kg bw / d (2.1 g/animal/day) being approximately equivalent to 150 mg/kg dry feed
NOPA and nitrate analysis of plasma	2016	n/a	4 Beef Cattle and 4 controls	29 days of 3 mg/kg bw (2g / animal) being equiv to 284 mg/kg (feed)
			28 beef cattle per dosing group	0,100,200 mg/kg feed for 238 days
Toxicity				
In-vitro Ames Microsuspension Test	2010	471	n/a	0, 1.6, 5, 15.8, 50, 158, 500 µg / plate, with and without S9 mix
In-vitro Salmonella typhimurium and Escherichia coli reverse mutation assay	2014	471	n/a	52, 164, 512, 1600 and 5000 µg/plate, with and without S9 mix
In-vitro Salmonella typhimurium and Escherichia coli reverse mutation assay II	2015	471	n/a	52, 164, 512, 1600, 5000 µg/plate (experiment I), 492, 878, 1568, 2800, 5000 µg/plate (experiment II) with and without S9 mix
Screening in-vitro Micronucleus Test in Chinese Hamster V79 Cells	2010	487	n/a	0, 310.8, 621.6, 1243.2 µg/mL (without S9-mix), 0, 77.7, 155.4, 310.8 µg/mL (with S9-mix)
In-Vitro V79 Micronucleus Assay	2020	487	n/a	0, 300, 480, 540, 570, 600 µg/mL (with S9-mix)
In-vitro Micronucleus assay in cultured peripheral human lymphocytes	2014	487	n/a	164, 512, 1211 µg/mL, with and without S9 mix

Study	Year	OECD	Animals	Doses tested
In-vitro mammalian cell gene mutation test (Mouse lymphoma assay)	2015	476	n/a	0, 0.55, 1.7, 5.4, 17, 52, 164, 512 and 1211 µg/mL, with and without S9 mix
Cell transformation (SHE) assay	2013	n/a (followed OECD draft proposal)	n/a	0, 500, 1000, 1500, 2000, 2250, 2500 µg/mL
In-Vitro TK6 Micronucleus Assay	2021	487	n/a	0, 750, 1000, 1220 µg/ml with and without S9 mix
Salmonella typhimurium and Escherichia coli reverse mutation assay **(NOPA)**	2020	471	n/a	**NOPA:** 0, 3,10,33,100, 333, 1000, 2500 and 5000 µg/plate with and without S9 mix
Micronucleus Test in Human Lymphocytes In vitro **(NOPA)**	2020	487	n/a	**NOPA:** 10.4,18.2,31.8,55.7,97.5,171,299,525,915,1372 µg/ml with and without S9 mix
Acute Oral Toxicity Test	2014	423	Wistar rats	300 - 2000 mg/kg bw
Assessment of acute inhalation toxicity	2017	436	Wistar rats	1 and 5 mg/L
Micronucleus test in bone marrow cells of the mouse (screening)	2011	474	NMRI Male mice (intraperitoneal)	0, 250, 500, 1000 mg/kg bw
Micronucleus test in bone marrow cells of the rat	2014	474	Wistar rats	0, 375, 750, 1500 mg/kg bw
10-day dose range finding study	2012	n/a	Wistar rats (n= 3 per group per sex)	0, 100, 300, 1000 mg/kg bw
Combined 28-day repeated dose toxicity study and reproduction / developmental toxicity screening test	2013	422, 407	Wistar rats	0, 10, 20, 100, 500 mg/kg bw
90-day oral gavage toxicity study	2015	408	Wistar rats	0, 50, 100, 300 mg/kg bw
Dose range finding study and the Maximum Tolerated Dose (MTD) study	2014	n/a	Beagle dogs, n = 2 (1 x M, 1xF) DRF, n = 2 per sex per dose MTD	25, 125 and 500 mg/kg bw (DRF) 0,30,100,200 (MTD study) mg/kg bw

Study	Year	OECD	Animals	Doses tested
14-day oral gavage toxicity study	2016	n/a	Beagle dogs 2 x M and 2 x F per dose	0, 150, 300 mg/kg bw (300 given as a split dose of 150 x 2, each 6 hours apart)
3-months oral gavage toxicity study	2016	409	Beagle dogs	0, 10, 30, 100, 300 mg/kg bw
1-year oral gavage toxicity study	2016	452	Wistar Rats	Males: 0, 25, 50, 100, 300 mg/kg bw Female: 0, 50, 100, 600 mg/kg bw
2-year carcinogenicity study	2019	451	Wistar Rats	Males: 0, 25, 50, 100 mg/kg bw Female: 0, 50, 100, 300 mg/kg bw
6-day DRF in mice	2018	451 and 417	CByB6F1 hybrid mouse	0, 124, 372, 742, 1224 mg/kg bw
28-day study in mice	2019	451	CByB6F1 hybrid mouse	0, 100, 300, 700 mg/kg bw
NOPA In-Vivo 14-Day Dose Range Finder Assay in Rats	2021	n/a	Fischer rats	**NOPA:** 0, 112, 335, 558 and 892 mg/kg bw/d (n=6, male), 0, 335, 670 and 1000 mg/kg bw/day (n=6, female)
NOPA In-Vivo Mutation Assay at the cII Locus and In-Vivo Micronucleus Assay in Male and Female Big Blue® Transgenic F344 Rats	2021	488, 474	Fischer rats	**NOPA:** 0, 150, 300 and 600 mg/kg/day (n=6_ male), 0, 250, 500 and 1000 mg/kg/day (n=6_female)
Reprotoxicity				
28-day oral gavage mechanistic study	2014	Based on 407	Wistar rats	0, 100, 300, 500 mg / kg bw
Prenatal developmental toxicity study	2015	414	Wistar Rats	0, 100, 300, 1000 mg/kg bw
Prenatal developmental toxicity study	2016	414	NZW Rabbits	0, 50, 150, 450 mg/kg bw
Two-generation reproduction study	2016	416	Wistar Rats	0, 25, 50, 100 (Male and Female), extra satellite group of females dosed at 600 mg/kg bw
6-10-day preliminary mechanistic study	2017	n/a	Wistar Rats (n=9 across the two dosing levels)	800 and 1000 mg/kg bw
Dose range finding (mechanistic)	2018	n/a	Wistar Rats (n=1 per dosing group)	3-NOP: 1000 mg/kg bw (Oral) NOPA (metabolite): 75,250,600 mg/kg bw (IV) HPA (metabolite): 75, 250, 400 mg/kg bw (IV) HPA: 75,250,350 mg/kg bw (SC)
Influence of metabolites on testicular toxicity in male rats, 10-day study	2018	n/a	Wistar Rats (n=5 per dosing group)	3-NOP: 800 mg/kg bw (Oral) NOPA: 425 mg/kg bw (IV) HPA: 350 then 250 (day 3 onwards) mg/kg bw (IV) HPA: 350 mg/kg bw (SC)

Study	Year	OECD	Animals	Doses tested
Single dose transcriptomics study	2017	n/a	Wistar rats (n= 8 per dosing group)	0, 100, 1000 mg/kg bw
Benchmark-Dose-Modelling	2019	n/a	n/a	n/a
In-vitro Steroidogenesis	2015	n/a	Human adrenal cells	0, 0.00001, 0.001, 0.01, 0.1, 1, 10 mM (3-NOP, NOPA and HPA)
Ex-vivo model testicular toxicity evaluation (**3-NOP, NOPA, HPA, inorganic nitrate**)	2015	n/a	Sprague Dawley rat	0, 0.002, 0.02, 0.5, 2 mM (all compounds)
Ex-vivo model testicular toxicity evaluation of **NOPA**	2016	n/a	Sprague Dawley rat	0, 0.02, 0.5, 2 mM (NOPA)
In-vitro / ex-vivo species comparison study using **NOPA**	2019	n/a	Testicular tissue from Wistar rats, Beagle dog, and Cynomolgus monkey (n=34 tissue samples for each species)	0,1,20,500,1200,2500 µM (NOPA)

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