GRAS Notice for Dried *Methylococcus capsulatus* Product

Prepared for:

Division of Animal Feeds (HFV-220)
Office of Surveillance and Compliance
Center for Veterinary Medicine
Food and Drug Administration
Dr. David Edwards
MPN 4, Room 2658
12225 Wilkins Avenue
Rockville, MD 20852

Submitted by:

Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington, DC 20001

On behalf of our client

Calysta, Inc.
1900 Alameda de las Pulgas
Suite 200
San Mateo, CA 94403United States

March 22, 2022

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Part 1 – Signed statements and certification

1.1 Applicability of 21 C.F.R. part 570, subpart E

We submit this Generally Recognized As Safe (GRAS) notice in accordance with 21 C.F.R. part 570, subpart E.

1.2 Name and address of the notifier

Company: Calysta, Inc.
Name: Tomas Belloso
Address: 1900 Alameda de las Pulgas
Suite 200
San Mateo, CA 94403
Phone: 314-368-7114

All communications on this matter are to be sent to Counsel for Calysta, Inc.

Melvin S. Drozen
Keller and Heckman LLP
1001 G Street, N.W., Suite 500 West
Washington, DC 20001
Phone: (202) 434-4222
Email: drozen@khlaw.com

1.3 Name of the notified substance

Dried *Methylococcus capsulatus* Product, hereinafter for ease of reference we will refer to the Dried *Methylococcus capsulatus* Product as FeedKind®. FeedKind® is a protein-rich single cell protein (SCP) intended for use as a feed ingredient in salmonid feed. FeedKind® is produced through the culture of methanotrophic and heterotrophic microbial consortia.

1.4 Applicable conditions of use of the notified substance

FeedKind® is intended for use as a protein source in salmonid species (*e.g.*, Atlantic salmon and rainbow trout) feed with maximum use levels based on published scientific studies and corroborating unpublished data at 18% FeedKind® in the diet.

1.5 Basis for the GRAS determination

Keller and Heckman LLP, on behalf of Calysta, Inc. hereby notifies the Agency of its determination that FeedKind® is Generally Recognized as Safe (GRAS), consistent with Section 201(s) of the Federal Food, Drug, and Cosmetic Act (FDCA). This GRAS conclusion is based on scientific procedures in accordance with 21 C.F.R. §§ 570.30(a) and (b).

1.6 Exclusion from premarket approval

FeedKind® is not subject to the premarket approval requirements of the FDCA based on our conclusion that the notified substance is GRAS when used as a protein source in salmonid feed.

1.7 Availability of data and information

The information for this GRAS conclusion, including analytical data, published studies, and information that are the basis for this GRAS determination, are available to FDA upon request as required by 21 C.F.R. § 570.225(c)(7)(ii)(A) or (B) by contacting Keller and Heckman LLP at the below address.

Melvin S. Drozen
Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington DC 20005
Tel: 202-434-4222
Fax: 202-434-4646
Email: drozen@khlaw.com

1.8 Applicability of FOIA exemptions

(b) (4)

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1.9 Certification

We certify on behalf of our client Calysta, Inc. that this GRAS conclusion is based on representative data from Calysta, Inc. required for the safety and GRAS status of FeedKind®. To the best of our knowledge, our GRAS Notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

(b) (6)

March 22, 2022

Melvin S. Drozen
Partner

Keller and Heckman LLP

Date

Part 2 – Identity, method of manufacture, specifications, and physical or technical effect

2.1 Scientific data and information that identifies the notified substance

FeedKind® is a biomass product of fermentation which utilizes a consortium of microorganisms to produce a high protein product for use in salmonid feed. The FeedKind® product has been thoroughly tested and characterized. **Figure 1** is a sample data sheet that will be included with the product listing chemical composition.

Figure 1: Label of FeedKind® Product

FeedKind®

For Further Manufacture of Feed

GUARANTEED ANALYSIS

Crude Protein	(min)	68.0	%
Crude Fat	(min)	5.0	%
Crude Fiber	(max)	1.0	%
Moisture	(max)	10.5	%
Ash	(max)	12.0	%

Ingredients: Dried *Methylococcus capsulatus* Product

Storage: Store in a dry and clean place at room temperature.

Directions: For further manufacture of feed for *Salmonidae* fish (18% maximum inclusion rate in final feed).

Lot#

Manufacturing Date:

Expiration Date: 12 months from manufacturing date

Manufactured by:

CALYSTA, Inc.
1900 Alameda de las Pulgas, Suite 200
San Mateo, CA USA 94403
Tel.: (650) 492-6880

Net Weight on Invoice

FeedKind® is the same substance described in the publicly available information supporting this GRAS notice, and referred to in the past as Bioprotein®. FeedKind® is produced by a process, under conditions, and in accordance with specifications that are essentially the same as for the production of the substance referred to as Bioprotein® and tested in the studies that are cited and summarized herein. Differences in the production of the Bioprotein® used in the toxicology and feeding studies and the laboratory scale and pilot-scale production of FeedKind® reflect changes in production parameters that optimize the full-scale production of this substance without significant alterations in the composition profile or quality of the product.

Specifically:

1. Bacterial production strains have been unchanged since the original filings with the European Food Safety Authority (EFSA) in the early 1990s.
2. Fermentation conditions (culture medium, temperature, etc.) are unchanged from those used by Norferm A/S[1] in the past, and Calysta continues to use many of the same protocols as Norferm A/S.
3. The nutritional, amino acid and mineral profile of the product produced on a pilot-scale in Wilton/Teesside, UK, is essentially identical to the profile of the substance produced on a laboratory scale in Menlo Park, CA, and to the profile of the substance produced by Norferm A/S, and identified as Bioprotein®, as shown by historical production data.
4. Product specifications have been consistent throughout the history of the production of FeedKind®, referred to in the past as Bioprotein®, attesting to the unchanged characteristics and quality the product regardless of production setting or scale.

The current FeedKind® manufacturing process and finished product characteristics remain essentially the same since its early development days in the 1980's and subsequent EU approval in 1995. The same four strains (archived at the NCIMB) have been used throughout the years. Recent genetic comparison studies have shown the current working cell banks used for production are unchanged from the originally deposited strains.

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[1] Dansk Bioprotein A/S was founded in 1985 in Odense, Denmark. Norwegian company Norferm DA, equally owned by Nycomed Amersham and Den norske Stats Oljeselskab (Statoil), was the majority shareholder in Dansk Bioprotein A/S. Dansk produced a protein-rich bacterial fermentation product for use in animal feed and marketed the product under the trade name BioProtein®. BioProtein® was initially approved in the European Union on July 30, 1995 for fattening pigs, calves, and fish. (Annex to Directive 82/471/EEC under the group 1.1 "Bacteria," a new item no. 1.1.2 "Bacteria cultivated on natural gas").

Nycomed exited its holdings in Norferm DA via a sale to DuPont in 1999. In 2006, DuPont and StatOil stopped pursuing the commercial development of BioProtein® and transferred the assets, technology, and strain bank to a new entity called BioProtein A/S. In 2014, Calysta acquired BioProtein A/S, which included the BioProtein brand, the fermentation technology to manufacture animal feed, and the strain bank from Norferm.

The manufacturing process, raw materials, and fermentation conditions remain unchanged with only slight technical improvements that help increase manufacturing efficiencies and safety. These slight technical improvements have not affected the composition of the finished product. Moreover, the finished product remains consistent when manufactured at different scales, because process, strains, nutrients, and fermentation conditions remain the same.

At all scales, the fermentation media is prepared with the same nutrients, the same production strains are used for the fermentation, growth and harvesting is done under the same conditions and time frames, harvested material is still centrifuged to increase dry matter, and the concentrate is still heat treated and subsequently dried.

The manufacturing scale has minimal effect on the finished product quality and composition. Changes in manufacturing efficiency might cause small variations in the finished product composition. However, the product quality remains the same despite the potential for small variations in some nutritional parameters.

Table 1 compares the composition of 3 batches of FeedKind® produced in 2020, as reported in Table 9, to the composition of the product used in safety studies, as submitted in the 1990s to the European Food Safety Authority (EFSA) for approval in the EU. Furthermore, Table 1 summarizes the recent and historical data, combined, and the data from published safety studies, including Storebakken *et al.* (2004) and Skrede *et al.* (1998). The data summarized in Table 1 show that the characteristics of the product, as reflected in the levels of major nutritional components and amino acid composition, are consistent across historical and current batches and regardless of production scale.

Table 1. Composition Profile of FeedKind® (aka Bioprotein®) Across Production Scales and Time				
Analyte	**Batches Reported in Table 9 (Mean ± SD*)[a]**	**Data Reviewed in Original EFSA Approval (Mean ± SD)[b]**	**All Batches (Recent and Lab Scale) (Mean ± SD)[c]**	**Product Composition Reported in Studies (Mean)[d]**
Crude Protein (g/100 g)	73.5 ± 0.75	70.6 ± 1.1	71.7 ± 1.8	68.2 (67.2%-69.2%)
Crude Fat (g/100 g)	8.5 ± 0.78	9.8 ± 0.57	9.3 ± 0.91	10.2 (10.2-10.3)
Crude Fiber (g/100 g)	0.07 ± 0.12	0.74 ± 0.31	0.49 ± 0.43	NR**
Moisture (g/100 g)	6.5 ± 0.33	5.7 ± 0.91	6.0 ± 0.81	2.7 (1.4-4.0)
Ash (g/100 g)	7.2 ± 0.75	7.1 ± 0.16	7.1 ± 0.42	7.8 (7.9-8.0)
Alanine (g/100 g)	5.2 ± 0.17	5.2 ± 0.09	5.2 ± 0.11	6.3 (6.2-6.4)
Arginine (g/100 g)	4.6 ± 0.09	4.4 ± 0.16	4.5 ± 0.19	5.4 (5.37-5.52)
Aspartic acid (g/100 g)	6.4 ± 0.31	6.5 ± 0.10	6.5 ± 0.19	7.9 (7.77-7.99)
Glutamic acid (g/100 g)	7.9 ± 0.20	7.7 ± 0.14	7.8 ± 0.18	9.3 (9.2-9.5)
Glycine (g/100 g)	3.7 ± 0.08	3.6 ± 0.06	3.6 ± 0.10	4.5 (4.41-4.54)
Histidine (g/100 g)	1.6 ± 0.07	1.8 ± 0.22	1.7 ± 0.21	1.8 (1.82-1.87)
Isoleucine (g/100 g)	3.2 ± 0.11	3.4 ± 0.16	3.3 ± 0.15	3.9 (3.8-3.9)
Leucine (g/100 g)	5.6 ± 0.19	5.5 ± 0.20	5.5 ± 0.19	7.2 (7.1-7.3)
Lysine (g/100 g)	4.2 ± 0.21	4.6 ± 0.09	4.4 ± 0.23	5.0 (4.89-5.03)
Phenylalanine (g/100 g)	3.1 ± 0.20	3.3 ± 0.13	3.2 ± 0.13	4.3 (4.22-4.34)
Proline (g/100 g)	2.7 ± 0.19	3.2 ± 0.42	3.0 ± 0.42	3.5 (3.45-3.55)
Serine (g/100 g)	2.4 ± 0.10	2.7 ± 0.11	2.6 ± 0.16	3.4 (3.36-3.45)
Threonine (g/100 g)	3.1 ± 0.16	3.3 ± 0.05	3.2 ± 0.13	4.0 3.93-4.04)
Tyrosine (g/100 g)	1.9 ± 0.16	NR	NR	3.2 3.16-3.25)
Valine (g/100 g)	4.1 ± 0.16	4.5 ± 0.17	4.3 ± 0.25	5.3 (5.27-5.42)
Tryptophan (Total) (g/100 g)	1.2 ± 0.12	1.6 ± 0.03	1.4 ± 0.21	1.8 (1.82-1.87)
Methionine (g/100 g)	2.0 ± 0.14	2.0 ± 0.08	2.0 ± 0.08	2.1 (2.11-2.17)
Cysteine +Cystine (g/100 g)	0.43 ± 0.03	0.45 ± 0.02	0.44 ± 0.03	0.78 (0.77-0.79)
Salt (from chloride) (g/100 g)	0.38 ± 0.08	0.76 ± 0.09	0.62 ± 0.21	NR
Ether Extract (g/100g)	1.4 ± 0.24	NR	NR	NR
Sodium (g/100 g)	0.35 ± 0.09	0.93 ± 0.20	0.71 ± 0.34	NR
Calcium (g/100 g)	0.27 ± 0.02	0.46 ± 0.08	0.39 ± 0.12	NR
Phosphorus (g/100 g)	1.7 ± 0.24	3.2 ± 0.64	2.6 ± 0.93	NR
Copper (mg/kg)	91.9 ± 18.0	90.6 ± 5.1	91.1 ± 10.4	NR
Zinc (mg/kg)	22.4 ± 7.7	17.0 ± 5.4	19.0 ± 6.5	NR
Manganese (mg/kg)	1.8 ± 1.6	<1	0.7 ± 1.1	NR
Iron (mg/kg)	355.7 43.4	216.2 ± 7.6	269 ± 76.0	NR
Magnesium (g/100 g)	0.32 ± 0.04	0.21 ± 0.02	0.25 ± 0.06	NR

*SD = standard deviation of the mean.

**NR = Not reported.

[a] n = 3 batches; 3 samples/batch; batches produced in 2020.

[d] n=2 batches; reported by Storebakken *et al.* (2004) and Skrede *et al.* (1998); all batches reported on wet basis.

[b] n=5 batches; 3 samples/batch.

[c] n= 8 batches.

2.2 Description of the method of manufacture of FeedKind®

2.2.1 Organisms

FeedKind® is produced through the culture of methanotrophic and heterotrophic microbial consortia. *Methylococcus capsulatus* (Bath) is a methanotrophic bacteria that composes 90% of the culture. It is a thermophilic bacterium originally isolated from the hot springs in Bath, England, is widely used as a laboratory culture and has been deposited as NCIMB 11132 at The National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland. *M. capsulatus* (Bath) has optimum growth at 45°C, but growth occurs between 37°C and 52°C. It is a gram-negative nonmotile spherical cell, usually occurring in pairs. The intracellular membranes are arranged as bundles of vesicular discs characteristic of Type I methanotrophs. *M. capsulatus* (Bath) is genetically a very stable organism without known plasmids. It can utilize methane or methanol for growth and ammonia, nitrate, or molecular nitrogen as nitrogen sources for protein synthesis. While only carbon sources containing a single carbon atom are utilized by *M. capsulatus* for growth (i.e., biomass), the organism is capable of oxidizing higher molecular weight hydrocarbons into their corresponding organic acid (e.g., ethane to acetic acid and propane to propionic acid). These higher molecular weight hydrocarbons are largely removed from the methane during processing of natural gas, but may remain in concentrations of 0-15% (ethane) to <5% (propane).

The FeedKind® culture includes three heterotrophic strains, *Cupriavidus sp.* (NCIMB 13287, previously *Alcaligenes acidovorans* DB3), *Aneurinibacillus danicus* (NCIMB 13288, previously *Bacillus brevis* DB4), and *Brevibacillus agri* (NCIMB 13289, previously *Bacillus firmus* DB5), all of which were isolated from mixed cultures growing on methane. The nomenclature changes are indicative of changes in the level of sophistication of bacterial taxonomy, and do not represent any changes to the actual strains utilized to produce FeedKind®. The nomenclature changes are due to a recent whole genome sequencing analysis of the three heterotrophic strains undertaken by the submitter in 2019 (**Appendix 1**). However, pending peer review and acceptance of these results, this strain will be referred to as *Cupriavidus sp*. in this submission. The sequence of DB4 indicated a 99.54% identity match to *Aneurinibacillus* UBA3580, which is a genome generated via metagenomic datasets, rather than from an actual isolate.[2] *Aneurinibacillus sp.* (NCIMB 13288) was previously renamed to *Aneurinibacillus danicus* based on 16s sequences, however no whole genome sequence was available at the time.[3] Finally, DB5 sequence analysis indicated a 99.56% identity match to *Brevibacillus agri*. Matches with greater than 95% nucleotide identity are considered extremely likely to be of the same species.

The purpose of the heterotrophic strains is threefold: 1) to metabolize organic acids (acetate, propionate, butyrate) produced by *M. capsulatus* that have been shown to reduce the efficiency with which *M. capsulatus* converts methane to biomass; 2) to metabolize organic material released via

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[2] Parks DH, Rinke C, Chuvochina M, Chaumeil P-A, Woodcroft BJ, Evans PN, Hugenholtz P, and Tyson GW. (2017) Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life. *Nature Microbiology* 2(11): 1533-1542.

[3] Goto K, Fujita R, Kato Y, Asahara M, Yokota A. (2004) Reclassification of *Brevibacillus brevis* strains NCIMB 13288 and DM 6472 (=NRRL NRS-887) as *Aneurinibacillus danicus* sp. nov. and *Brevibacillus linophilus* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*. 54 (2):419-427.

naturally occurring cell lysis during fermentation which could lead to foam formation; and 3) to minimize the risk of culture contamination by undesirable microbes.

Previous regulatory submissions used old taxonomy for the heterotrophic strains, all three of which have been reclassified utilizing modern molecular techniques. However, the strains used have not changed from those previous submissions.

1) *Cupriavidus sp.* (NCIMB 13287) is a gram-negative, aerobic, motile rod. It can utilize ethanol, acetate, propionate and butyrate for growth. *Cupriavidus sp.* accounts for 6-8% of the total cell count of a FeedKind® culture grown on natural gas.
2) *Aneurinibacillus danicus* (NCIMB 13288) is a gram-positive, endospore-forming, aerobic rod. It can utilize acetate, D-fructose, D-mannose, ribose and D-tagatose. It accounts for less than 1% of the cell count during continuous fermentation.
3) *Brevibacillus agri* (NCIMB 13289) is a gram-positive, endospore-forming, motile, aerobic rod. It can utilize acetate, N-acetyl-glucosamine, citrate, gluconate, D-glucose, glycerol and mannitol. It accounts for less than 1% of the cell count during continuous fermentation.

2.2.2 FeedKind® Production

(b) (4)

Figure 2: FeedKind® Production Process

Table 2: Process Temperature and Residence Times

Operation	Operating Temperature	Residence Time
Fermenter	(b) (4)	
Centrifuge		
Heat Kill		
Evaporator		
Spray Dryer		

2.2.2.1 Microbial Hazard Control

As described in more detail in section 2.2.2 above, FeedKind® is produced through the culture of the methanotrophic bacteria Methylococcus capsulatus and the three heterotrophic microbial consortia consisting of Cupriavidus *sp.* (DB3), *Aneurinibacillus danicus* (DB4), and *Brevibacillus agri* (DB5), all of which were isolated from mixed cultures growing on methane. *Methylococcus capsulatus*, the main strain used in the manufacturing process, requires very specific conditions that need to be consistently maintained for the microbe to survive and proliferate. During (b) (4) (b) (4) (b) (4) (*see* Table 2), one of which has been validated to be a true (b) (4) (b) (4) and is the only CCP in Calysta's HACCP, and as such, it has been validated and constantly monitored and verified.[4] All FeedKind® fermentation production organisms are destroyed or inactivated under normal operating conditions. Calysta's internal studies have proven the heat kill to be effective at reducing the viable bacterial load by 4.5-9.1 log of magnitude (*see* Table 3). Finished product analysis shows that none of the heterotrophic production microorganisms are viable in the finished product.

Calysta carried out a heat kill validation study of the (b) (4) (b) (4)

[4] Heat treatment of (b) (4) is globally recognized across feed, food, and pharma industries and has been documented extensively in peer reviewed scientific literature to be an effective means to destroy or kill bacterial species.

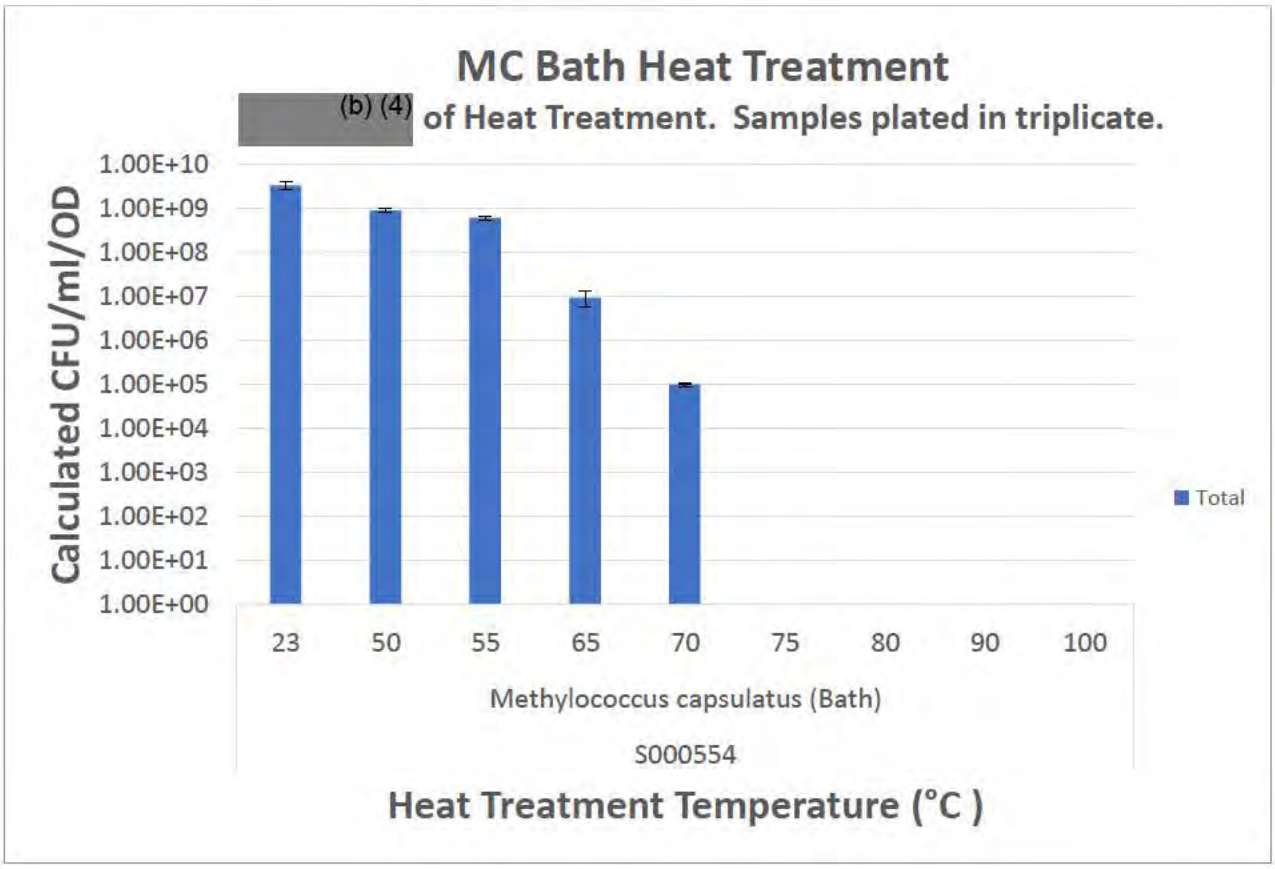
Table 3. UHT Heat Kill Validation Data

	Median	
	Pre- Heat treatment	**Post Heat Treatment**
DB3	(b) (4)	
DB4/5		
TVC		

The total plate count method utilized for testing of each batch allows for enumeration of both remaining heterotrophic organism from the production run as well as any potential contaminating microorganisms. This method is based on EN ISO 4833:2013 and is validated for use in animal feed. See Appendix 8 for the method summary. (b) (4) (b) (4) (b) (4)hat tested Calysta's sample lots, utilizes an (b) (4)method based on EN ISO 4833:2013 which also utilizes (b) (4) a growth medium. Typical time and temperature i (b) (4) (b) (4)

(b) (4) (i.e. methane or methanol) for growth. *M. capsulatus* is difficult to culture in a way which would easily permit enumeration on a per lot basis. To address this, Calysta has developed internal data which indicates that *M. capsulatus* is entirely inactivated by the heat treatment process employed during production. Figure 3 shows that *M. capsulatus* is inactivated in as (b) (4) As described above, Calysta's process includes a heat kill step of (b) (4) The included heat kill data combined with the total plate count specification clearly indicates that the conditions of the manufacture for FeedKind® reduce the number of viable production organisms by more than 5 logs (>99.999%).

Figure 3: *M. capsulatus* Heat Kill Curve



Calysta has previously tested 276 separate lots of FeedKind® (finished product) produced during research and development phases to optimize the production process. Of these lots, 8 results were extremely high (> 300,000 cfu/g due to laboratory handling errors) and were excluded from further analyses. The average of the remaining lots is ~31,000 cfu/g with a standard deviation of ~82,000. All of these production lots were produced using an (b) (4). As part of Calysta's drive for continued improvement, the (b) (4) (b) (4) (b) (4) (b) (4) (b) (4)

[5] The heat treatment data provided in this submission supersede the data included within AGRN 40 and accompanying supplemental data, submitted by Calysta, Inc. on August 13, 2020, which were generated using the previously utilized UHT system and are no longer relevant to the current manufacturing process.

Table 4. Microbial Analysis on Product Heat Treated with (b) (4) (b) (4)

BATCH NUMBER	Aerobic Plate Count (TVC) (CFU/g)	Salmonella (/25 g)	Listeria species (/25 g)	Yeasts (CFU/g)	Molds (CFU/g)	Enterobacteriaceae (CFU/g)
TPP-019/01	(b) (4)					
TPP-019/02						
TPP-019/03						
TPP-019/04						
TPP-019/05						
TPP-019/06						
TPP-019/07						
TPP-009/08						
TPP-019/09						
TPP-019/10						
TPP-019/11						
TPP-019/12						
TPP-019/13						
TPP-019/14						

BATCH NUMBER	Aerobic Plate Count (TVC) (CFU/g)	Salmonella (/25 g)	Listeria species (/25 g)	Yeasts (CFU/g)	Molds (CFU/g)	Enterobacteriaceae (CFU/g)
TPP-019/15	(b) (4)					
TPP-019/16						
TPP-019/17						
TPP-019/18						
TPP-019/19						
TPP-019/20						
TPP-019/21						
TPP-019/22						
TPP-019/23						
TPP-019/24						
TPP-019/25						
TPP-019/26						
TPP-019/27						
TPP-019/28						
TPP-019/29						

BATCH NUMBER	Aerobic Plate Count (TVC) (CFU/g)	Salmonella (/25 g)	Listeria species (/25 g)	Yeasts (CFU/g)	Molds (CFU/g)	Enterobacteriaceae (CFU/g)
TPP-019/30	(b) (4)					
TPP-019/31						
TPP-019/32						
TPP-019/33						
TPP-019/34						
TPP-019/35						
TPP-019/36						
TPP-019/37						
TPP-019/38						
TPP-019/39						
TPP-019/40						
TPP-019/41						
TPP-019/42						
TPP-019/43						
TPP-019/44						

BATCH NUMBER	Aerobic Plate Count (TVC) (CFU/g)	Salmonella (/25 g)	Listeria species (/25 g)	Yeasts (CFU/g)	Molds (CFU/g)	Enterobacteriaceae (CFU/g)
TPP-019/45	(b) (4)					

The safety of the production microorganisms and large reduction in viable cell counts due to post fermentation processing indicate that FeedKind® is not expected to pose any microbiological safety concerns. Any bacterial count is the result of environmental contamination or poor sampling technique. As with all food production systems the Calysta system operates in a non-sterile environment where some non-pathogenic bacterial growth is to be expected. As such, Calysta has built a final product specification based on current capabilities, that would ensure the safety of the product.

Measurement	Specification
Aerobic Total Viable Count (TVC)	<10,000 CFU/g
Yeasts	<1,000 CFU/g
Molds	<1,000 CFU/g
Salmonella	Absent in 25 g
Listeria	Absent in 25 g

Fermentation conditions used in the process are unfavorable for growth of pathogenic microorganisms. While total plate count can be used to gauge sanitary quality of a product, Calysta has tested over 391 samples for salmonella and listeria over the last 50 months since the manufacturing plant initiated its operations. All samples tested showed no *Salmonella* or *Listeria* contamination which is indicative of the fact that the manufacturing process is effective at minimizing the potential risk of pathogen contamination. Additionally, a review of the most similar previous animal GRAS notices (i.e. microbial biomass ingredients) indicates that Calysta's specification is not out of line with previous notices for which CVM has provided "no questions" letters.

As discussed more thoroughly in Part 6.1.1 of this notice, the organisms which are used to produce FeedKind® are safe for consumption. The main production organism, *M. capsulatus,* is a methanotroph that requires single carbon energy sources (e.g., methane or methanol) for growth, as well as elevated temperatures. Furthermore, *M. capsulatus* is not known to produce any toxins and there are no literature reports of pathogenicity in humans or animals. For these reasons,

methanotrophs are not considered to be risks for pathogenicity in animals or humans. Finally, the three heterotrophic strains were tested in rodents and exhibited no ability to cause infections, even at very high doses (>10^9 cfu/kg bw).

FeedKind® is a fine dry powder with a water activity (a_w) level of (b) (4) and therefore is microbiologically shelf stable in ambient conditions. Test results from shelf-life samples from 5 different lots retained in December 2018 and then retested in July 2021 (>30 months) show a maximum water activity of (b) (4) is lower than the required a_w of more than 0.8 to support microbial growth. (*see* Table 5).

It is important to mention that the heat treatment is performed whilst the process material is still in (b) (4) form with a (b) (4). Treating the material at this stage of the process reduces the risk of low a_w associated thermal resistance in pathogenic bacteria occurring in the final dried product.[6]

Table 5. Water Activity and Microbial Testing in Retained Samples

Batch Date (Date Dried)	Batch Reference	At Time of Production (CFU/g)		After 30 Months (CFU/g)			
		Total Viable Count	Yeasts & Molds	Total Viable Count	Yeasts & Molds	pH	Water Activity
05-Dec-18	TEES009/88	(b) (4)					
07-Dec-18	TEES009/99						
18-Dec-18	TEES009/100						

[6] Cho W. and Chung M. (2020) Bacillus spores: A review of their properties and inactivation processing technologies. *Food Sci Biotechnol*. 29(11): 1447-1461; Leuschner R.G.K and Lillford P.J (1999) Effects of temperature and heat activation on germination of individual spores of *Bacillus subtilis*. *Letters in Applied Microbiology*. 29: 228–232; Syamaladevi R.M et al. (2006) Influence of water activity on thermal resistance of microorganisms in low-moisture foods: a review. *Comprehensive reviews in food science and food safety*. 353-370; Silva et al. (2013) Methods of destroying bacterial spores. Microbial pathogens and strategies for combating them: science, technology and education (A. Méndez-Vilas, Ed.) pp490-496; Xu S, Labuza T.P, and Diez-Gonzalez F. (2006) Thermal Inactivation of *Bacillus anthracis* Spores in Cow's Milk. Appl. Environ. Microbiol. p4479–4483.

18-Dec-18	TEES009/101	(b) (4)
18-Dec-18	TEES009/102	

Pathogens[7]				
Batch Date (Date Dried)	Batch Reference	At Time of Production (CFU/g)		After 30 Months (CFU/g)	
		Salmonella	*Listeria*	*Salmonella*	*Listeria*
05-Dec-18	TEES009/88	(b) (4)			

[Remainder of page intentionally left blank]

[7] Additional pathogen testing was conducted on batch TEES09/88. Calysta has set a specification for these pathogens, and included testing for same in multiple batches (see **Tables 8 and 9)**.

2.2.2.2 Raw materials and processing aids

All raw materials and processing aids, including fermentation media components, utilized in the production of FeedKind® are safe and suitable for use in feed production, and are prepared and handled as feed ingredients. These materials are listed in **Table 6**.

Table 6. Raw Materials and (Example) Processing Aids

Raw Material	Function	Authorization Reference	Authorization Limits	Specification	Units	Value
Methane & Natural Gas	Nutrient for culture	None; Safe for use[8]	N/A	(b) (4)		(b) (4)
Ammonium Hydroxide	pH control	21 CFR §582.1139	Good Manufacturing or Feeding Practice (GM/FP)		NA	
					wt%	
Sulfuric Acid	pH control	21 CFR §582.1095	GM/FP		N/A	
					wt%	
Phosphoric Acid	Nutrient for culture	21 CFR §582.1073	GM/FP		N/A	
					wt%	
					ppm	
					ppm	
					ppm	
					ppm	
					ppm	

[8] *See* discussion in Section 2.2.2.3 of this GRAS Notice.

Sodium Hydroxide	pH control	21 CFR §582.1763	GM/FP	(b) (4)	NA wt% ppm ppm ppm	(b) (4)
Potassium Hydroxide Solution	Nutrient for culture	21 CFR §582.1631	GM/FP		NA wt% wt% wt% ppm ppm ppm	
Zinc Sulfate Heptahydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary supplements		N/A wt% ppm ppm ppm	
Nickel Chloride	Nutrient for culture	None; Safe for	N/A		N/A	

Hexahydrate		use[9]		(b) (4)	wt% ppm ppm ppm ppm ppm	(b) (4)
Cobalt Sulfate Heptahydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary		N/A wt% ppm ppm ppm ppm	
Manganese Sulfate Monohydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary		wt% ppm ppm ppm ppm	

9 *See* discussion in Section 2.2.2.4 of this GRAS Notice.

Nitric Acid[10]	pH control and Nutrient for culture	None; GRAS[11]	N/A	(b) (4)		(b) (4)
					wt%	
					wt%	
Copper Sulfate Pentahydrate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary		NA wt% ppm ppm ppm ppm	
Sodium Molybdate Dihydrate	Nutrient for culture	AAFCO Definition # 57.145	N/A		 wt% ppm ppm ppm ppm ppm	
Iron Sulfate	Nutrient for culture	21 CFR §582.80	GFP; added as nutritional dietary		NA wt%	

[10] Nitric acid is stored in opaque (stainless steel) containers and is not stored in direct sunlight. Therefore, possible photochemical reactions will not take place and there is no related safety concern.

[11] *See* Section 2.2.2.5 of this GRAS Notice.

				(b) (4)	wt%	(b) (4)
					wt%	
					wt%	
					wt%	
					wt%	
					wt%	
Calcium Chloride Solution (Prepared From Calcium Chloride Dihydrate In De-Mineralized Water)	Nutrient for culture	21 CFR §582.1193	GM/FP			
					wt%	
					wt%	
					wt%	
					wt%	
					wt%	
					wt%	
Magnesium Sulfate Solution (Prepared From Magnesium Sulfate Heptahydrate	Nutrient for culture	21 CFR §582.5443	GM/FP; used as a nutrient and/or dietary supplement			
					wt%	
					ppm	

In De-Mineralized Water And Sulfuric Acid) (Note: this can be a substitute for Manganese Sulfate Monohydrate)				(b) (4)	ppm ppm ppm	(b) (4)
(b) (4) **Antifoam**	Antifoam	21 CFR §§ 172.808, 173.340, 582.4505	*See* Section 2.2.2.6		NA wt%	

[Remainder of page intentionally left blank]

All ingredients and processing aids in **Table 6**, including those that rely on 21 CFR Part 582 for an appropriate regulatory status, will be used in accordance with good manufacturing and feeding practice.

FeedKind® will be distributed in lined polypropylene bags, which are widely used within the animal feed industry and which have appropriate regulatory status under 21 CFR § 177.1520.

The current natural gas specifications for FeedKind® are listed in **Table 7**.

Table 7. Current Natural Gas Specifications

Natural Gas	Units	Specification
Nitrogen	Mol %	0-0.5
Methane	Mol %	82-100
Ethane	Mol %	0-15
Propane	Mol %	0-5
I-Butane	Mol %	0-2
N-Butane	Mol %	0-2
I-Pentane	Mol %	0-0.7
N-Pentane	Mol %	0-0.7
Calorific value – volume	Mol %	37.7-44
Benzene	ppmv	NMT 40
Mercury	µg/Nm^3	NMT 0.02

NMT = Not More Than

All FeedKind® production ingredients and processing aids that are not listed in the Code of Federal Regulations (CFR), Title 21, the subject of a GRAS Notification, or an AAFCO definition, are provided below, which includes a discussion of the intended use and safety of each substance in the manufacture of FeedKind®.

2.2.2.3 Natural gas constituents safety

Natural gas is composed primarily of methane, although ethane, propane, butane and pentane may also be present.[12] Before refinement, natural gas may also contain 0% to 5% hydrogen sulfide (H_2S) gas, elemental mercury (Hg^0) vapor, and noble gases, such as argon (A), helium (He), neon (Ne), or xenon (Xe). After refining, natural gas is essentially methane, with low levels of ethane, and propane. None of these gaseous or vaporous natural gas constituents are expected to remain in finished FeedKind®. Methane serves as a food source for the bacteria and most, if not

[12] NATURALGAS.ORG. Available at: https://web.archive.org/web/20140709040340/http://naturalgas.org/overview/background/.

all, of the methane and other residual gaseous or vaporous substances that may enter the system with the refined pipeline natural gas and may not be dissolved or otherwise incorporated into the fermentation mix, is vented out of the system during the fermentation, centrifugation, evaporation and re-circulation processes. Furthermore, any residuals of these gases and vapors that may remain in the harvested wet biomass before spray drying will dissipate away from the product during spray drying.

Aromatic hydrocarbon constituents of natural gas, including benzene and toluene, as well as cyclohexane, are potentially subject to induced-dipole to induced-dipole interactions with the aromatic amino acids of proteins and, thus, may have the potential to remain in finished FeedKind® at detectable levels. No benzene or related compounds are expected to be in FeedKind®. However, this potential is discussed in Section 2.2.2.3.1 and evaluated in detail in Appendix 2.

Hg^0 is extremely volatile. Thus, no Hg^0 is expected in Feedkind®. However, some of the Hg^0 that may be present in the refined natural gas used to produce FeedKind® may be metabolized by the bacteria to produce methylmercury (MeHg) during fermentation. MeHg has the potential to bioconcentrate in the bacteria and remain in the product after spray-drying. This potential is discussed in Section 2.2.2.3.2 below and evaluated in detail in Appendix 3.

2.2.2.3.1 n-Hexane, cyclohexane, benzene and toluene safety

Natural gas contains benzene, toluene, and cyclohexane, which can be hypothesized to remain in finished FeedKind® because of induced-dipole-to-induced-dipole interactions with the aromatic amino acid constituents of FeedKind®. The toxicology of these constituents and n-hexane is well characterized in the published scientific literature, and well-established, generally-accepted toxicity values are available for all of them.[13] This enables conservative assessment of safety, assuming that detectable levels of these substances may remain in the finished product through induced-dipole-to-induced-dipole interactions.

We performed a screening-level safety assessment assuming:

- The natural gas used in the production of FeedKind® invariably contains the highest level of each of these constituents reported in the extensive survey of Chao and Attari (1995),[14] which equaled or exceeded the corresponding concentrations reported more recently by Black & Veatch (2021).[15]
- None of these constituents are eliminated through evaporation from the fermenter or metabolism by the bacterial consortium (despite published evidence to the contrary) and, therefore, 100% of n-hexane, cyclohexane, benzene and toluene that enters the fermenter with natural gas during fermentation are present in the FeedKind® biomass after centrifugation.
- 98.95% of the benzene, cyclohexane, and n-hexane will evaporate with water during the subsequent evaporation and spray-drying steps because the boiling points and vaporization enthalpies of these substances are lower than the corresponding values for water.

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13 n-Hexane | IRIS | US EPA; Cyclohexane (CASRN 110-82-7) | IRIS | US EPA; Benzene (CASRN 71-43-2) | IRIS | US EPA; Toluene (CASRN 108-88-3) | IRIS | US EPA

14 Chao and Attari (1995), Figure 1, page 12.

15 Black & Veatch (2021). Natural Gas technical Paper. Prepared for Calysta, 7 pp.

- No loss of toluene, in an abundance of caution, because the boiling point of benzene is 10% greater than the boiling point of water.
- Salmonids have the capacity to metabolize, and thereby detoxify, these constituents rapidly and to a significant extent, based on data from published studies.
- High-end exposures were estimated for human consumption of salmon and trout raised on diets containing 18% FeedKind®

Additional assumptions included:

- Cumulative feed consumed by the target animal per weight of edible tissue (i.e. 1.77 and 2.14 kg feed/kg edible body weight for Atlantic salmon and trout, respectively)[16]
- 100% of the intake of each constituent from the feed accumulates in the edible fish tissue
- High chronic daily consumption of salmon or trout by humans is equal to the 90th percentile daily ingestion level of all finfish (i.e., 0.17 kg/day)[17]
- Body weight 70 kg[18]

The results clearly show the upper bound cancer risk estimate for benzene is less than 10^{-6} (i.e. *de minimis*), and all HQs for all natural gas constituents would be orders of magnitude less than 1 even at the maximum concentrations of constituents reported in natural gas and exaggerative worst case exposure assumptions. Thus, there is no reasonable expectation of harm associated with the consumption of salmonids fed FeedKind® up to the maximum 18% inclusion level in salmonid food.

This assessment is presented in greater detail in the **Appendix 2**.

2.2.2.3.2 Mercury safety

Natural gas often contains trace levels of mercury (Hg), predominantly elemental mercury (Hg^0), which must be removed from the gas phase before transport via pipeline to protect downstream heat exchangers from catastrophic failures and catalysts from fouling. The concentrations of Hg in pipeline natural gas is reduced to concentrations below <0.01 µg/Nm^3 by means of current industry practices in the U.S., and Calysta specifications require that natural gas used to produce FeedKind® will contain no more than 0.02 µg/Nm^3. Inorganic Hg can be metabolized by microorganisms to produce methyl mercury (MeHg), which can then bioaccumulate in the food chain to result in bioconcentration factors (BCFs) as high as 80,000 in fish at the top of the food chain in aquatic environments.

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16 *See* Table 2 in Fry JP, Mailloux NA, Love DC, Milli MC, Cao L (2018). Feed conversion efficiency in aquaculture: do we measure it correctly? Environ. Res. Lett. 13: 024017: https://iopscience.iop.org/article/10.1088/1748-9326/aaa273/pdf.

17 *See* Table 2.055 in Smiciklas-Wright H, Mitchell DC, Mickle SJ, Cook AJ, Goldman JD (2002). USDA 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII 1994-1996).

18 NRC (2005) specifies an MRL of 0.0003 mg Hg/kg bw/day for a 70-kg person

The toxicity of Hg compounds has been well characterized in the literature. MeHg is generally more toxic than inorganic forms of Hg and is of particular concern because it also has the greatest potential to bioaccumulate. The NRC (2005) determined that salmon tolerate chronic MeHg up to 1 mg/kg diet and set a maximum tolerable level of 0.3 µg/kg bw/day for pregnant women to protect children in utero from MeHg from maternal fish ingestion.

A conservative upper limit (and specification) for the Hg concentration in FeedKind® was calculated for the protection of human health based on the MRL for human exposure, assuming that salmon or trout are raised on feed containing the maximum proposed use level of FeedKind® (i.e., 18%), exclusively, and that 100% of the Hg in FeedKind® is in the form of MeHg. Additional assumptions included:

- Cumulative feed consumed by the target animal per weight of edible tissue (i.e., 1.77 and 2.14 kg feed/kg edible body weight for Atlantic salmon and trout, respectively)[19]
- 100% of the Hg intake from the feed accumulates in the edible fish tissue
- High chronic daily consumption of salmon or trout by humans is equal to the 90th percentile daily ingestion level of all finfish (i.e., 0.17 kg/day)[20]
- Body weight 70 kg[21]

Based on these exaggerative assumptions, the upper limit of the Hg concentration in salmon or trout is 10 µg/kg (i.e., 0.01 mg/kg), which was derived as follows.

- **General Equation:** MRL (µg/kg bw/day) x bw (kg) ÷ [Edible Fish Tissue Consumption (kg/day) x Fish Feed Consumed per Edible Tissue Produced (kg/kg)] x Maximum Feedkind® Concentration in Fish Feed (%) = Hg Concentration Limit in FeedKind® (µg/kg)
- **For Salmon:** 0.3 µg/kg bw/day x 70 kg ÷ (0.17 kg x 1.77 kg/kg) x 18% = 12.6 µg/kg
- **For Trout:** 0.3 µg/kg bw/day x 70 kg ÷ (0.17 kg x 2.14 kg/kg) x 18% = 10.4 µg/kg

Rounding down from the lowest of these values (i.e., 10.4 µg/kg) yields a limit of 10 µg/kg for Hg in KeedKind®.

For the protection of the health of the fish, NAS (2005) specified an MTL of 1 mg/kg (i.e. 1000 µg/kg) for MeHg in the diet (but no MTL for inorganic Hg), which is two orders of magnitude greater than the 10 µg/kg specification derived for total Hg in FeedKind®.

We calculated exaggerative estimates of the maximum Hg concentrations based on an example FeedKind® production scenario. In that scenario, (b) (4) FeedKind® are produced in a single-fermenter production system where a substantially greater faction of the culture medium than required is re-circulated into the production system over each 12-week fermentation cycle. After each fermentation cycle, the system is emptied, cleaned, and prepared to

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19 *See* Table 2 in Fry JP, Mailloux NA, Love DC, Milli MC, Cao L (2018). Feed conversion efficiency in aquaculture: do we measure it correctly? Environ. Res. Lett. 13: 024017: https://iopscience.iop.org/article/10.1088/1748-9326/aaa273/pdf.

20 *See* Table 2.055 in Smiciklas-Wright H, Mitchell DC, Mickle SJ, Cook AJ, Goldman JD (2002). USDA 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII 1994-1996).

21 NRC (2005) specifies an MRL of 0.0003 mg Hg/kg bw/day for a 70-kg person

receive fresh bacterial culture and medium to start the next 12-week cycle. The worst-case assumptions incorporated into these calculations include the use of natural gas that invariably contains the maximum concentration of Hg, 100% conversion of the Hg to MeHg by the bacterial consortium, and BCFs as high as 80,000 for MeHg during fermentation.

We also estimated worst-case human exposures to Hg in the edible tissues of salmon and trout fed FeedKind®, assuming that the fish are fed exclusively food containing the maximum level of FeedKind®, all of the Hg in FeedKind® accumulates exclusively in the edible tissues of the fish, consumers eat only salmon or only trout fed exclusively food containing the maximum level of FeedKind®, and that high-end consumers eat only salmon or only trout at the same daily rate as estimated for the consumption of all finfish, combined, by high-end consumers (i.e. 90th percentile) of finfish.

The worst-case maximum concentration of Hg in salmonid feed containing FeedKind® at the highest use level was 0.00936 µg/kg, which is 106,838 times lower than the concentration tolerated by salmon determined by NRC (2005). The worst-case exposures to Hg were estimated to be 0.0000389 µg/kg bw/day and 0.0000486 µg/kg bw/day for exclusive consumers of salmon and trout, respectively, which are 6175 to 7719 times lower than the maximum tolerable level set by NRC (2005). Thus, the results of these exaggerative exposure calculations demonstrate the there are no significant risks to the target animals or to consumers of the edible tissues of the salmon or trout raised exclusively on food containing FeedKind® at the highest use levels.

This assessment is presented in greater detail in the **Appendix 3**.

2.2.2.4 Nickel chloride hexahydrate safety

Nickel is required in very small amounts (b) (4) finished product) as a nutrient in the fermentation media. As the production process for FeedKind® involves re-circulation and reuse of some water recovered from the fermentation media, it is theoretically possible that nickel could accumulate in the fermentation media over time resulting in higher concentrations of nickel in the final finished product. Although this has not been observed in any production run to date, a specification of 10 ppm nickel in the finished product was established.

Nickel from nickel chloride hexahydrate

As discussed in NRC (2005), nickel is an essential element for some lower forms of life. For example, nickel is essential for nitrogen metabolism in plants and for the activity of hydrogenases identified in more than 35 species of bacteria, including nitrogen-fixing bacteria.[22] However, nickel is not considered to be essential for higher animals and humans, although experimental nickel deprivation has been shown to result in subnormal functions that appear to be associated with vitamin B_{12} activity.

[22] National Research council (NRC) (2005). Nickel. Chapter 22 in: Mineral tolerance of animals. Committee on Minerals and toxic Substances in diets and water for Animals, Board on agriculture and Natural resources, Division on Earth and Life Studies, Second Revised Edition, pp. 276-283.

Typically, less than 10% of the nickel in food is absorbed in the gastrointestinal tracts of animals and humans, and the small amount that is absorbed is excreted mostly in the urine (NRC 2005).

Nickel compounds are known carcinogens by inhalation exposure, and nickel is a recognized allergen by respiratory and dermal exposure. Allergic effects are possible in sensitized individuals exposed to high levels of nickel in the diet. However, there is no evidence of adverse health effects in humans associated with chronic dietary exposure to nickel. The tolerable upper intake level for humans is 0.017 mg/kg bw/day, based on a NOAEL of 5 mg/kg bw/day in two rat studies (NRC 2005).

In animal studies, the first signs of nickel toxicity appear to be the result of reduced food intake, which is attributable at least in part to reduced palatability of the diet, and gastrointestinal irritation. The most common signs reported after extended exposures include reduced growth, feed intake and feed efficiency, as well as hematological changes and sometimes renal effects. Elevated incidences of the death of offspring have been reported in developmental and reproductive toxicity studies in rats and chickens exposed to soluble nickel salts in drinking water or the diet, indicating the potential for impaired reproductive performance. Some of the effects of long-term excessive oral exposures to nickel are attributable to interference by nickel with the gastrointestinal absorption or use of essential elements, including copper, iron, and zinc, which are more evident when the diet is deficient in these elements. Alterations in cellular redox status, resulting in excessive levels of reactive oxygen species, has also been suggested as a potential mechanism of nickel toxicity (NRC 2005).

On the other hand, the potential for life-threatening toxicity is considered to be low, similar to zinc, chromium, and manganese, for example, because of the existence of effective homeostatic mechanisms for the regulation of nickel.

Generally, toxicity has been observed in animal studies only after chronic exposures to more than 100 ppm water-soluble nickel in the diet of rats, mice, chickens, dogs, rabbits, pigs, ducks, and monkeys. NRC (2005) suggested maximum tolerable levels of dietary nickel of 100 ppm for cattle, 250 ppm for chickens and pigs, and approximately 1000 ppm for dogs (NRC 2005).

Most plant-based animal feeds contain relatively high concentrations of nickel and animal-tissue-based feeds contain comparatively low concentrations. Langmyhr and Orre (1980) reported substantial concentrations of 0.7 to 2.8 ppm nickel in five different fish protein concentrates considered for use as a source of protein and trace elements in human nutrition.[23] However, most animal feeds contain less than 10 ppm nickel, which is an order of magnitude less that the lowest maximum tolerated dose of 100 ppm suggested by NRC (2005). Accordingly, Maule et al. (2007) reported an average nickel concentration of 2.35 ppm, ranging from 0.42 to 7.8 ppm, in 55 fish feed samples collected from 11 National Fish Hatcheries between October 2001 and October 2003.[24]

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[23] Langmyhr FJ, Orre S (1980). Direct atomic absorption spectrometric determination of chromium, cobalt and nickel in fish protein concentrate and dried fish solubles. Analytica Chimica Acta 118: 307-311.

[24] Maule AG, Gannam AL, Davis JW (2007). Chemical contaminants in fish feeds used in federal salmonid hatcheries in the USA. Chemosphere 67: 1308-1315.

EFSA (2015) reviewed several studies of nickel in fish feed, which support a NOAEL of 10 ppm nickel in feeds for salmonid species.[25] In particular, Ptashinsky et al. (2001 and 2002) reported a LOAEL of 100 ppm and a NOAEL of 10 ppm in Lake Whitefish fed diets supplemented with water-soluble nickel for 10, 31, or 104 days.[26] Histopathological changes in the kidneys were found in the fish fed diets containing ≥100 ppm in this study. In another study, Javed (2013) reported decreased weight gain, fork length, and feed intake in Major Carp fed 73 ppm water-soluble nickel (*i.e.* the lowest concentration tested) for 12 weeks.[27] In addition, Alsop et al. (2014) reported reduced growth in male and reduced total egg production in female Zebrafish fed 116 ppm water-soluble nickel (*i.e.* the lowest concentration tested) for 80 days; Zebrafish are commonly used as an animal model for aquaculture nutrition research.[28]

By comparison, the concentration of nickel would be 1.8 ppm in a salmonid diet containing 18% FeedKind® that contains the specified maximum concentration of 10 ppm nickel. This level (*i.e.* a maximum of 1.8 ppm) is well below the NOAEL of 10 ppm in salmonid species and the maximum tolerable level of 100 ppm suggested by NCC (2005) for domestic animals. Further, comparison with the results published in Maule et al. (2007) indicate that the inclusion of up to 1.8 ppm nickel in fish feed via the inclusion of FeedKind® - used as a replacement for other protein sources that might be in fish feed – is likely to result in fish-feed nickel concentrations below the average of 2.35 ppm nickel reported in fish feeds.

Chloride from nickel chloride hexahydrate

As discussed in NRC (2005), chloride is an essential nutrient for essentially all forms of life, most notably because chloride, along with sodium, is critical for maintaining osmotic and acid-balance.[29] The bodies of nearly all animal species maintain extracellular and intracellular osmotic concentrations between 250 and 400 milliosmoles (mOsm). Saltwater fish live in water that typically contains 1,000 mOsm, most of which is attributable to sodium chloride in the water. About 77% of the total dissolved solids in saltwater is composed of sodium chloride. Most saltwater fish excrete sodium and chloride through the gills to maintain normal levels of water in their bodies against the osmotic

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[25] EFSA (2015). Scientific Opinion on the risks to animal and public health and the environment related to the presence of nickel in feed. EFSA Journal 13(4): 4074 (59 pp.)

[26] Ptashynski MD, Pedlar RM, Evans RE, Baron CL, Klaverkamp JF (2002). Toxicology of dietary nickel in lake whitefish (Coregonus clupeaformis). Aquatic Toxicology 58: 229–247; Ptashynski MD, Pedlar RM, Evans RE, Wautier KG, Baron CL, Klaverkamp JF (2001). Accumulation, distribution and toxicology of dietary nickel in lake whitefish (Coregonus clupeaformis) and lake trout (Salvelinus namaycush). Comparative Biochemistry and Physiology. Toxicology & Pharmacology 130: 145–162.

[27] Javed M (2013). Chronic effects of nickel and cobalt on fish growth. International Journal of Agriculture & Biology 15: 575–579.

[28] Alsop D, Santosh P, Lall, SP, Wood CM (2014). Reproductive impacts and physiological adaptations of zebrafish to elevated dietary nickel. Comparative Biochemistry and Physiology C 165: 67–75.

[29] National Research council (NRC) (2005). Sodium chloride. Chapter 27 in: Mineral tolerance of animals. Committee on Minerals and toxic Substances in diets and water for Animals, Board on agriculture and Natural resources, Division on Earth and Life Studies, Second Revised Edition, pp. 357- 371.

pressure exerted by the high salt concentration of the water around them. The gills pump sodium against the concentration gradient of the saltwater and chloride follows sodium out of the body.

In contrast, the kidneys of freshwater species excrete very dilute urine to reduce the loss of salt and maintain normal levels of body water against the tendency of the water to diffuse from extracellular fluids to the surrounding freshwater. Freshwater fish typically also have efficient mechanisms for absorbing sodium and chloride from water through the gills. NRC (2005) notes that higher sodium chloride concentrations in saltwater can reduce the toxicity of minerals such as mercury, cadmium, chromium, and zinc, by competing with and reducing the uptake of these minerals through the gills. Accordingly, freshwater fish are generally more sensitive to nitrite than saltwater fish because the chloride ions in saltwater compete with nitrite for absorption through the gills.

As discussed in NRC (2005), excessive amounts of chloride added to the diet as a component of trace metals used in very high amounts to supplement the diet, apart from sodium, has the potential to acidify extracellular fluids, resulting in metabolic acidosis.[30] However, the trace elements in most diets are absorbed in such small amounts that the possibility of altered acid-base status is negligible. In any case, mild acid-base imbalances are amenable to correction through increased renal excretion of cations or anions. Sodium chloride added to the diet generally has essentially no effect on acid-base physiology.

NRC (2005) notes that freshwater fish do not tolerate water containing more than 1500 ppm sodium chloride and saltwater fish do not survive in water containing more than 30,000 ppm.

As noted by Salman (2008), dietary sodium chloride up to 11.6% (*i.e.* 116,000 ppm) did not impair the growth of rainbow trout when isonitrogenous/isocaloric diets were used.[31] Dietary sodium chloride levels of 1% to 4% (*i.e.* 10,000 to 40,000 ppm) have been demonstrated to have beneficial effects in salmon, carp, trout, and other freshwater fish species, and are commonly used in diets for salmon and trout.[32]

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30 National Research council (NRC) (2005). Minerals and Acid-base Balance. Chapter 33 in: Mineral tolerance of animals. Committee on Minerals and toxic Substances in diets and water for Animals, Board on agriculture and Natural resources, Division on Earth and Life Studies, Second Revised Edition, pp. 449-452.

31 Salman NA (2009). Effect of dietary salt on feeding, digestion, growth and osmoregulation in teleost fish. Chapter 4 In: Osmoregulation and Ion Transport, Volume 1, Handy, Bury and Flick, eds., Society of Experimental Biology UK (SEB).

32 *See, e.g.*, Salman NA, Eddy FB (1988). Effect of dietary sodium chloride on growth, food intake and conversion efficiency in Rainbow trout (Salmo gairneri Richardson). Aquaculture 70: 131-144; Mzengereza K, Kang'ombe J (2015). Effect of salt (sodium Chloride) supplementation on growth, survival and feed utilization of Oreochromis shiranus (Trewavas, 1941). J. Aquac. Res. Develop. 7(1): 3 pp.

As noted, small amounts of nickel chloride hexahydrate are added as a nutrient to the fermentation medium, which is, thus, present at up to 2 ppm[33] in finished feed. Based on the literature reviewed above, salmonid species tolerate, and even benefit from, dietary chloride concentrations several orders of magnitude greater than the potential contribution of chloride from nickel chloride in FeedKind®. Therefore, the chloride from nickel chloride hexahydrate has no potential to adversely affect the health of these species when fed FeedKind®.

2.2.2.5 Nitric acid safety

Nitric Acid is a source of nitrogen and a pH control agent. The term nitrate (NO_3^-) refers to salts and esters of nitric acid (HNO_3). As noted in NRC (2005), nitrates are formed naturally in the biological nitrogen cycle (nitrification), through which ammonia in the soil is oxidized by aerobic bacteria to produce nitrite and then nitrate.[34] Plants then use the nitrate to synthesize amino acids and proteins. In contrast, nitrates are not essential nutrients for mammalian species.

Like nitrites (NO_2^-), nitrates are rapidly absorbed in the intestines of nonruminant mammals and the rumen of ruminants.[35] The plasma half-life of nitrate ranges from 4.2 to 4.8 hours in sheep and ponies and up to 44.7 hours in dogs.[36] Nonruminant animals generally excrete more urinary nitrate than ruminants.

Nitrate itself is not highly toxic. However, nitrate has the potential to be converted to nitrite, which can oxidize hemoglobin in the bloodstream to produce methemoglobin. Unlike hemoglobin, methemoglobin cannot transport oxygen. In ruminants, bacteria in the rumen rapidly convert nitrate to nitrite and nitrite to ammonia, and the ammonia is used by the bacteria to synthesize amino acids and proteins.[37] Thus, nitrate toxicity in ruminants occurs only when the conversion of nitrite to ammonia is disrupted or the nitrate levels in the diet are high enough to saturate the conversion process in the rumen. In nonruminants, methemoglobin in is usually associated with the consumption of high levels of nitrite rather than nitrate.

The clinical signs of acute methemoglobinemia may be evident when 30% to 40% of the hemoglobin in the bloodstream is converted to methemoglobin, including rapid breathing and pulse

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[33] We acknowledge that there are other sources of chloride in the fermentation media, but these are from sources and in uses that are permitted at levels consistent with good manufacturing and feeding practices. The issue addressed in this section is whether the chloride potentially added as part of the nickel chloride hexahydrate poses a health risk to the animals to which it is being fed.

[34] National Research council (NRC) (2005). Nitrates and nitrites. Chapter 34 in: Mineral tolerance of animals. Committee on Minerals and toxic Substances in diets and water for Animals, Board on agriculture and Natural resources, Division on Earth and Life Studies, Second Revised Edition, pp. 453-468.

[35] Walker R (1990). Nitrates, nitrites and N-nitroso compounds: a review of the occurrence in food and diet and the toxicological implications. Food Addit. Contam. 7(6): 717-768.

[36] NRC (2005).

[37] Russell JB (2002). Rumen microbiology and its role in ruminant nutrition. Cornell University, New York state college of Agriculture and Life Sciences, Department of Microbiology, Ithaca NY.

rate, muscle tremors, and increased urination.[38] Methemoglobinemia may be fatal at methemoglobin levels greater than 80%. The effects of chronic nitrate exposure are difficult to detect in ruminants because these animals can use nitrate as a nitrogen source. However, abortions have been reported in ruminants receiving doses of nitrate high enough to cause clinical signs of toxicity. Reduced feed intake has been reported in beef cattle and sheep receiving more than 10,000 ppm and 30,000 ppm dietary nitrate. Other possible effects mentioned in the literature include methemoglobinemia, placental transfer of methemoglobin, changes in pituitary function, transfer of some nitrate to milk, and effects on vitamin A metabolism in ruminants chronically exposed to nitrate or nitrite. However, as NRC (2005) notes, accumulation of nitrates and nitrites is not expected in the tissues of animals or in the milk of mammals because these substances are generally excreted rapidly.

As reviewed in NRC (2005), reduced body weight gains among nonruminants were reported in chickens and rats fed 3,100 ppm and 2,916 ppm nitrate in the diet, respectively. Increased fetal losses have been reported in guinea pigs treated with nitrate. However, no effect has been observed on the reproductive performance of pigs, and reports of such responses in chickens have been inconsistent.

NRC (2005) suggested a maximum tolerable level of 1,823 ppm nitrate in the diet based on the results of rat studies. In comparison, ruminants exposed to more than 5,000 ppm nitrate in the diet (dry matter basis) have exhibited signs of toxicity.

NRC (2005) suggested that the EPA guideline of 10 ppm nitrate-N (*i.e.* 44 ppm NO_{3-})[39] in drinking water is a conservative maximum tolerable level because some studies have found no effects in animals exposed to 200 times this guideline and others have reported reduced animal performance only at 20 times the guideline. In comparison, the NRC (1974) recommended an upper limit of 100 ppm nitrate-N (*i.e.* 442 ppm NO_3^-) in the drinking water of livestock and poultry.[40]

Nitrate is generally much less toxic to fish and other aquatic organisms than is nitrite.[41] Jensen (1999) noted that the mechanism of toxicity in fish is generally the same as in terrestrial animals, specifically the oxidation of hemoglobin to methemoglobin.[42] The effects of nitrite toxicity in fish include reduced growth rates and suppressed immune function. Nitrite has also been studied

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38 NRC (2005).

39 10 ppm nitrate-N x 4.42 grams NO_3^-/gram nitrate-N = 44.2 ppm NO_3^-.

40 NRC (1974). Nutrients and Toxic Substances in water for Livestock and Poultry. National academy Press, Washington D.C. (cited by NRC 2005).

41 Basuyaux O, Mathieu M (1999). Inorganic nitrogen and its effect on the growth of the abalone Haliotis tuberculata Linneaus and the sea urchin Paracentrotus lividus Lamark. Aquaculture 174: 95-107; Colt J, Tchobanoglous G (1976). Evaluation of the short-term toxicity of nitrogenous compounds to channel catfish, Ictalurus punctatus. Aquaculture 8: 209-224; Pierce RH, Weeks JM, Prappas JM (1993). Nitrate toxicity to five species of marine fish. J. World Aquac. Soc. 24: 105-107 (all cited in NRC 2005).

42 Jensen FB (1999). Physiological effects of nitrite in teleosts and crustaceans. In: Toxicology of Aquatic Pollution Physiological, Molecular and Cellular Approaches, Taylor EW, ed. Cambridge, UK: Cambridge University Press, pp. 169-186 (cited in NRC 2005).

for its possible role in the formation of mutagenic and carcinogenic N-nitroso compounds, which have been detected in the muscle and other tissues of nitrite-exposed rainbow trout, for example.

Freshwater fish are generally more sensitive to nitrite than saltwater fish because the chloride ions in saltwater inhibit the uptake of nitrite by competing with nitrite for absorption through the gills. Jensen (1999) noted that fish species with high branchial chloride uptake rates, including rainbow trout, perch, and pike, appear to be more sensitive to nitrite toxicity than species with low uptake rates, such as carp, tench, and eel. However, exposure to nitrite concentrations in the millimolar range can be tolerated by fish for long periods if the water chloride concentrations are elevated sufficiently.

By comparison, the toxicity of nitrate is very low in most aquatic species, with ambient nitrate concentrations of several tens of millimolars required to increase mortality in short-term toxicity tests. Camargo et al. (2005) noted that the relatively low toxicity of nitrate, compared to nitrite and ammonia, is attributable at least in part to the low branchial permeability of nitrate.[43] These authors also noted that long-term exposure to nitrate at the EPA guideline of 10 ppm nitrate-N for drinking water can adversely affect freshwater fish, including Rainbow trout, Cutthroat trout and Chinook salmon. Camargo et al. (2005) cited Kincheloe et al. (1979), who reported elevated mortality of the larvae of these species in water containing 2.3 to 7.6 ppm nitrate-N (*i.e.* 10 to 33.6 ppm, NO_3^-).[44] Based on their review of the literature, Camargo et al. (2005) recommended 2 ppm nitrate-N (*i.e.* 8.8 ppm NO_3^-) as a maximum water concentration to protect the most sensitive freshwater species and 20 ppm nitrate-N (*i.e.* 88 ppm NO_3^-) as a likely maximum water concentration for the protection of saltwater species. However, Freitag et al. (2016) found no difference in survival of Atlantic salmon embryos exposed to mean nitrate-N levels of 4 or 93 ppm (*i.e.* 17.7 and 411 ppm NO_3^-).[45]

Davidson et al. (2014) reported that rainbow trout exposed to 80 to 100 ppm nitrate-N (*i.e.* 354 to 442 ppm NO_3^-) for three months demonstrated chronic health and welfare impacts including an increase in abnormal swimming behavior, increased swimming speeds, and mildly reduced survival.[46]

Based on the results, these authors recommended 75 ppm nitrate-N (*i.e.* 332 ppm NO_3^-) as the upper design limit for water recirculating aquaculture systems used for rainbow trout culture. However, Davidson et al. (2017) reported no effects of chronic (8 months) exposure to 100 ppm nitrate-N (*i.e.* 442 ppm NO_3^-) on survival, swimming behavior or any other measures of a

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43 Camargo JA, Alonso A, Salamanca A (2005). Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. Chemosphere 58: 1255–1267.

44 Kincheloe JW, Wedemeyer GA, Koch DL (1979). Tolerance of developing salmonid eggs and fry to nitrate exposure. Bull. Contam. Toxicol. 23: 575-578.

45 Freitag AR, Thayer LR, Leonetti C, Stapleton HM, Hamlin HJ (2015). Effects of elevated nitrate on endocrine function in Atlantic salmon, Salmo salar. Aquaculture 436: 8-12.

46 Davidson J, Good C, Welsh C, Summerfelt ST (2014). Comparing the effects of high vs. low nitrate on the health, performance, and welfare of juvenile rainbow trout Oncorhynchusmykiss within water recirculating aquaculture systems. Aquacultural Engineering, 59: 30-40.

comprehensive set of health variables in post-smolt Atlantic salmon.[47] Davidson et al. (2017) concluded that post-smolt Atlantic salmon can be cultured humanely in aquaculture systems in which the mean nitrate level is maintained at or below 100 ppm nitrate-N (*i.e.*, 442 ppm NO_3^-).

As noted above, nitric acid serves as a nitrogen source and pH control agent in the culture medium used to support the bacterial growth and protein synthesis on which the production of FeedKind® depends. The final concentration nitrate in FeedKind® resulting from nitric acid added to the medium at the start of bacterial growth period is expected to be negligible at the end of this period primarily because most, if not essentially all, of the nitrate will be consumed by the bacteria to synthesize amino acids and proteins. In any case, salmonid species are clearly tolerant of nitrate-N concentrations in water at least up to 2 ppm (*i.e.* 8.8 ppm NO_3^-), and likely at much higher concentrations (*i.e.* up to 100 ppm nitrate-N; 442 ppm NO_3^-), depending on the salinity of the water, life-stage of the fish, and other factors evaluated in the published scientific literature reviewed above. Thus, the contribution of any residual nitrate in FeedKind® resulting from the use of nitric acid in the culture medium to the overall exposure of the fish to nitrates will be negligible in recirculating aquaculture systems operated in accordance with good aquaculture practice.

Information from Calysta's nitric acid supplier regarding the heavy metals analysis of the nitric acid ingredient is provided in Appendix 4. While this does not constitute a "specification" *per se*, it does indicate that for mercury, arsenic, cadmium, and lead, the concentration is below detection limits (<0.1 ppm for arsenic, cadmium, and lead, 0.005 ppb for mercury). Iron is present at very low levels (0.23 ppm) however this is a negligible amount of iron in comparison to the iron added to the fermentation media (as iron sulfate) which is a required nutrient for the growth of the microbes. Nitric acid is used at a rate of approximately 0.07 mg per kg of finished feed, with iron being present in the nitric acid at 0.23 ppm. Iron sulfate is added to ensure an iron concentration of 300-350 ppm, and therefore any potential contribution to the overall amount of iron present from nitric acid (0.23 ppm in the nitric acid) is negligible.

2.2.2.6 Defoamer authorization

Methylobacterium extorquens protein ("*M. extorquens*" or "KnipBio Meal") appears, per the unredacted portions of AGRN 00026, to use an ethylene oxide-propylene oxide block (EO-PO) copolymer defoamer. *See* AGRN26, **Appendix 2**. The manufacturer of the defoamer used in the manufacture of KnipBio Meal attested to the fact that the defoamer is authorized for use in human food under 21 CFR § 173.340 and that FDA has taken an enforcement discretion policy for the use of defoamers authorized for use in human food when used in the manufacture of animal feed. *Id.*, AGRN 00026 **Appendix 3** (the "Yingling Letter"). We understand that defoamers listed in the Yingling Letter must meet other specifications on the list and, given FDA's letter of no objection, we presume, although we cannot confirm, that the defoamer used in the production of KnipBio Meal was on the lists in the Yingling Letter and complied with the supplemental information – for EO-PO copolymers the Yingling Letter matches the listing in 21 CFR § 172.808, including CAS number (9003-11-6).

[47] Davidson J, Good C, Williams C, Summerfelt ST (2017). Evaluating the chronic effects of nitrate on the health and performance of post-smolt Atlantic salmon Salmo salar in freshwater recirculation aquaculture systems. Aquacultural Engineering 79: 1-8.

There is no practical difference between the defoamer used by KnipBio and that used by Calysta (b) (4) - the defoamer used by Calysta in the fermentation of Feedkind® is also an EO-PO copolymer the manufacturer of which attests that its use is authorized under 21 CFR §§ 172.808 and 173.340 (including that the CAS number is 9003-11-6).

Beyond the EO-PO copolymer, (b) (4) also incorporates rapeseed oil and fatty acids from rapeseed oil, both of which are identified as GRAS by the manufacturer. Mono- and diglycerides of rapeseed oil (CAS 93763-31-6) are on the Yingling list and mono- and diglycerides of edible fats or oils and edible fat forming acids are permitted for use in animal feed for use as emulsifying agents consistent with good manufacturing and feeding practice, 21 CFR § 582.4505. It is well established and recognized that triglycerides are metabolized into mono- and diglycerides as well as fatty acids – so the presence of rapeseed triglycerides (a precursor) and fatty acids from rapeseed oil (a product) are as safe and suitable for use as a defoamer as are rapeseed oil mono- and diglycerides.

We therefore conclude that (b) (4) is safe and suitable for use as a defoamer in the manufacture of FeedKind®.

2.2.2.7 Heavy metal safety

With the exception of the components already identified as being used pursuant to established regulatory authorizations (*e.g.*, copper and zinc) or which are safe for use at the levels contemplated (*e.g.* nickel), there is no appreciable risk that heavy metals will be present in the finished product as none of the inputs into the fermentation media are expected to contain heavy metals at above negligible levels.[48] FeedKind® distributed in the United States will be produced domestically, and therefore mercury contamination from the natural gas feedstock is not expected to occur. Previous testing conducted by Calysta to fully characterize FeedKind® indicates that in most cases mercury is below the detection limit (<0.01 ppm) and in no cases was above 0.03 ppm.

[48] There may be mercury present in natural gas as it leaves the ground, but, on the basis of the US EPA risk assessment, we do not expect mercury to be present in the natural gas used to manufacture FeedKind®. US EPA (2001) Mercury in petroleum and natural gas: estimation of emissions from production, processing, and combustion. Available at: https://cfpub.epa.gov/si/si_public_record_report.cfm?Lab=NRMRL&dirEntryId=63480.

This risk assessment of mercury found in petroleum and natural gas in the United States acknowledges that, while mercury is a natural component of natural gas, removal strategies are employed and residual mercury in US pipeline gas was present at a negligible level (<0.03-0.3 ppb). As levels of mercury in the raw materials used in the media are orders of magnitude lower than the recommended permissible level of 2 ppm from the AAFCO "Official Guidelines for Contaminant Levels Permitted in Mineral Feed Ingredients," Table 2, there is no cause for concern from mercury in the product. AAFCO, 2018 Official Publication, "Official Guidelines for Contaminant Levels Permitted in Mineral Feed Ingredients," Table 2, located at page 298.

2.3 Specifications for FeedKind®

The FeedKind® product has been thoroughly tested and characterized. The specifications for FeedKind® are included in **Table 8.**

Table 8. FeedKind® Specifications

Specifications				
Chemical Composition	**Minimum**	**Maximum**	**Units**	**Test Method**
Crude Protein	(b) (4)		% dry weight	(b) (4) method
Crude Fat			% dry weight	modified Weibull Acid Hydrolysis Method
Crude Fiber			% dry weight	AOCS Ba 6a-05, Ba 6-84 AOAC 962.09, S 1022 using Gravimetry
Ash[49]			% dry weight	AOAC 942.05, S 1024 using Gravimetry
Moisture			w/w	AOAC 934.01, 930.15, S 1024 using Gravimetry

[49] Appendix 5 contains analyses of 289 lots. These data show that a true average value for (b) (4) Ash fluctuates predictably due to fermentation stage and productivity. Startup and low productivity levels in the fermenter deliver higher ash level while high productivity or steady state operations have lower ash levels. The representative samples had low ash because they happened to be taken during periods of high productivity. Given that ash is primarily salts and minerals present in the media, and higher ash is not seen as a health risk because Calysta concurrently monitors for heavy metals and contaminants directly, we believe that leaving the ash specification at (b) (4). Ash is not used as a proxy for any other measurements.

Nickel		(b) (4)	*mg/kg*	ICP-MS Internal Method[50]
Mercury			*mg/kg*	ICP-MS Internal Method[51]
Microbiological Limits		**Limits**		**Test Method**
Mesophilic Aerobic Plate Count[52]		(b) (4)		EN ISO 4833:2013
Mold				AOAC 997.02; FDA/BAM Chapter 18
Yeast				AOAC 997.02; FDA/BAM Chapter 18

[50] (b) (4) has developed an in-house validation method for the detection measurement of nickel with an LOD of 0.1mg/kg in animal feed. The method and validation summary are included in Appendices 6 and 7. Appendix 8 details (b) (4) validation of various analytical methods.

[51] The method used to determine the mercury content is validated and accredited by (b) (4) The method summary is included in Appendix 8 for detection of mercury at an LOD of 0.01mg/kg.

[52] Calysta has previously tested 276 separate lots of FeedKind® produced during research and development phases to optimize the production process, the results of which were previously provided to FDA. Of these lots, 8 results were extremely high (> 500,000 cfu/g). Reference to these production lots have been removed as they were produced with the old production process prior to introduction of the new direct steam treatment and as such are not relevant to this notice.

Salmonella	(b) (4)	EN ISO 6579-2:2017
Listeria		EN ISO 11290-1:2017

Analysis of three (3) non-consecutive batches of FeedKind® can be found in **Table 9**. Test methods for the analysis are listed in **Table 8**, above.

Table 9. Result of Batch Analysis for 3 Non-Consecutive Batches

BATCH NUMBER	TEES-009/63	TEES-009/102	TEES-009/84
Crude Protein (g/100 g)	(b) (4)		
Crude Fat (g/100 g)			
Crude Fiber (g/100 g)			
Moisture (g/100 g)			
Ash (g/100 g)			
Nickel (mg/kg)			
Mercury (mg/kg)			
Aerobic Plate Count (TVC) (cfu/g)			
Molds (cfu/g)			
Yeasts (cfu/g)			
Salmonella (/25 g)			
Listeria species (/25 g)			
Bacillus cereus (cfu/g)			
***Escherichia coli* (cfu/g)**			
Pepsin digestibility (%)			
Alanine (g/100 g) [53]			
Arginine (g/100 g)			
Aspartic acid (g/100 g)			
Glutamic acid (g/100 g)			
Glycine (g/100 g)			
Histidine (g/100 g)			
Isoleucine (g/100 g)			
Leucine (g/100 g)			
Lysine (g/100 g)			
Phenylalanine (g/100 g)			

[53] Amino acid content determined using AOAC 994.12.

Proline (g/100 g)	(b) (4)
Serine (g/100 g)	
Threonine (g/100 g)	
Tyrosine (g/100 g)[54]	
Valine (g/100 g)	
Tryptophan (Total) (g/100 g)[55]	
Methionine (g/100 g)	
Cysteine +Cystine (g/100 g)	
Salt (from chloride) (g/100 g)	
Ether Extract (g/100g)	
Sodium (g/100 g)	
Calcium (g/100 g)	
Phosphorus (g/100 g)	
Copper (mg/kg)	
Zinc (mg/kg)	
Manganese (mg/kg)	
Iron (mg/kg)	
Magnesium (g/100 g)	

The reported composition for the three submitted lots accounted for 93.9%-94.6% of the mass. The unaccounted 5%-6% is primarily composed of soluble carbohydrates. Calysta has developed laboratory scale data indicating that typical carbohydrate content is approximately 8%. A summary of the lab scale test is provided here:

(b) (4)

[54] A true determination of tyrosine would require a second, separate analysis which would incur significant expenses. Tyrosine numbers reported are those derived from AOAC 994.12, even though it is not strictly appropriate for this determination. We believe this is of little consequence as tyrosine content is not directly safety related. See Appendix 9 for tyrosine testing data.

[55] See Appendix 8 for methods and validation information for tryptophan.

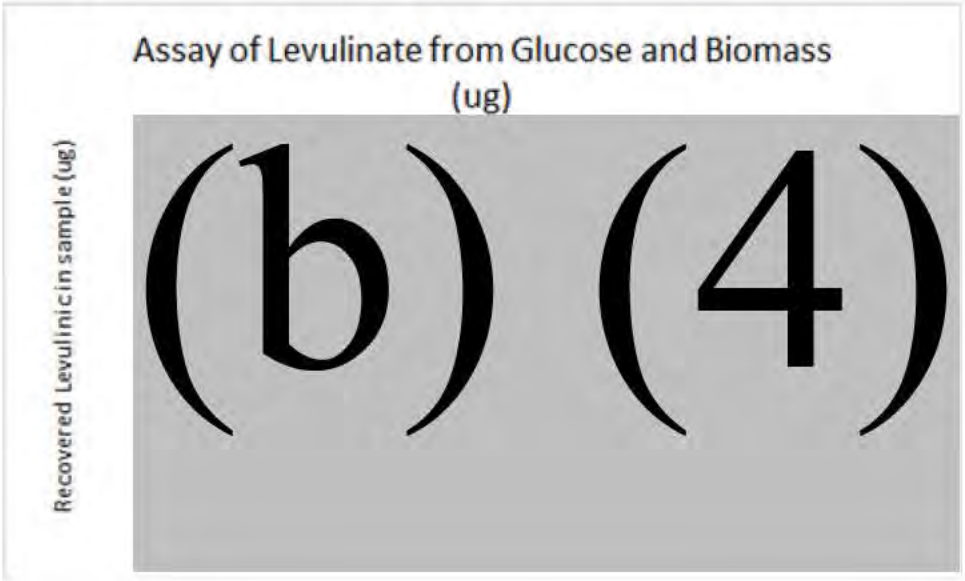
(b) (4)

.

Table 10. Summary of Lab Scale Test

1 OD pellet (ug)	Glucose Spike (ug/ sample)	Theoretical Levulinate from Glucose (ug)	Levulinate from pellet (ug)	Theoretical Levulinate from Glucose and Biomass (ug)	Assay of Levulinate from Glucose and Biomass (ug)
350	(b) (4)				
350					
350					
350					
350					

Figure 4: Assay Levulinate from Glucose and Biomass (ug)



While Calysta does not directly test for carbohydrates (beyond fiber), the remaining product not accounted for by the current specifications is expected to be carbohydrates, and this is supported by the laboratory scale data above. Because of the nature of the product, the expected inaccuracy of available methods, and because of FeedKind®'s intended function (sources of protein), we believe it to be unnecessary develop and validate a method for such an analysis. For nitrogen, the (b) (4) method employed by Calysta to determine "crude protein" will indeed report results for all nitrogen containing compounds such as nucleic acids and biogenic amines. From the previously submitted stability results, we know that biogenic amine content ranges from 3,000-5,000 ppm (0.3-0.5%). The remaining nitrogen content is comprised of nucleic acids. While Calysta does not assay

for nucleic acid content, this level is consistent with a microbial biomass products in general and FeedKind® specifically as illustrated in Skrede *et al.* 1998, which indicates a nucleic acid content of ~9.5%.[56] Further, this would not present a safety concern to animals and would be broken down in the animal's digestive tract and not passed into the human food supply.

With regards to the mineral content, phosphorus is reported as elemental phosphorus. When accounting for the fact that phosphorus is typically present as phosphate (PO_4), it accounts for approximately (b) (4) of product. Chloride is reported as salt content. Molybdenum is expected to be present in FeedKind® at approximately (b) (4) based on microbial media usage rates, and therefore we do not believe analysis for molybdate content is required. In sum, the data from the batch analyses, including the measured levels of crude protein, individual amino acids, phosphorus (as phosphate), other minerals, glucose and carbohydrates containing glucose, together with reported levels of nucleic acids in the published scientific literature, account for nearly 100% of the composition of FeedKind®.

2.4 Potential contaminant concentration testing

During production runs, Calysta conducts daily testing for compositional analyses (e.g. protein, ash, moisture, etc.) as well as periodic (approximately weekly) testing and monitoring of the FeedKind® product. This periodic testing includes analyses of potential heavy metal contaminant concentration in the continuous fermentation run, including for lead (Pb), cadmium (Cd), and arsenic (As). Test results from three separate continuous fermentation runs (i.e., TEES-004/1-59, TEES-005/01-54, and TEES-009/1-102) are provided below in Table 11, which summarizes the first, last, and range of analytical results obtained from the three fermentation runs. Samples are numbered sequentially (i.e. TEES-004/1 is from Day 1, and TEES-04/59 is from day 59). Results for TEES-09 begin after an initial experimental period of 33 days in which the early batches were not subjected to a heat kill step. These batches were deemed to be not suitable due to undesirable microbial growth and were discarded. The heat kill step is included in the production process for all commercial batches. Subsequent batches, for which results are reported in Figure 7, were subjected to the heat kill step. Results for mercury (Hg) and nickel (Ni) are also provided, where available.

Table 11. Summary of Heavy Metal Concentrations During Continuous Fermentation Runs

TEES-004/1-59					
Analyte (mg/kg)	**Pb**	**Cd**	**As**	**Hg**	**Ni**
First	(b) (4)				
Last					
Range	0.015-0.032	0.001-0.006	0.006-0.017	<0.001-0.003	N/A

[56] Skrede A, Berge GM, Storebakken T, Herstad O, Aarstad KG, Sundstol F (1998). Digestibility of bacterial protein grown on natural gas in mink, pigs, chicken, and Atlantic salmon. Animal Feed Sci. Technol. 76(1-2): 103-116.

TEES-005/01-54					
First	(b) (4)				
Last					
Range	<0.09-0.11	<0.02-<0.02	<0.05-<0.05	<0.01-0.03	1.12-1.89
TEES-009/1-102					
First	(b) (4)				
Last					
Range	<0.09-<0.11	<0.02-0.03	<0.05-0.051	<0.01	1.94-5.25

Figures 5-7 plot the concentration of the specified heavy metals during the three continuous fermentation runs.

Figures 5-7: Concentration of heavy metals during three separate continuous fermentation runs. Nickel concentrations in Figure 6 and Figure 7 are plotted on the right axis.

Figure 5 – Run 1

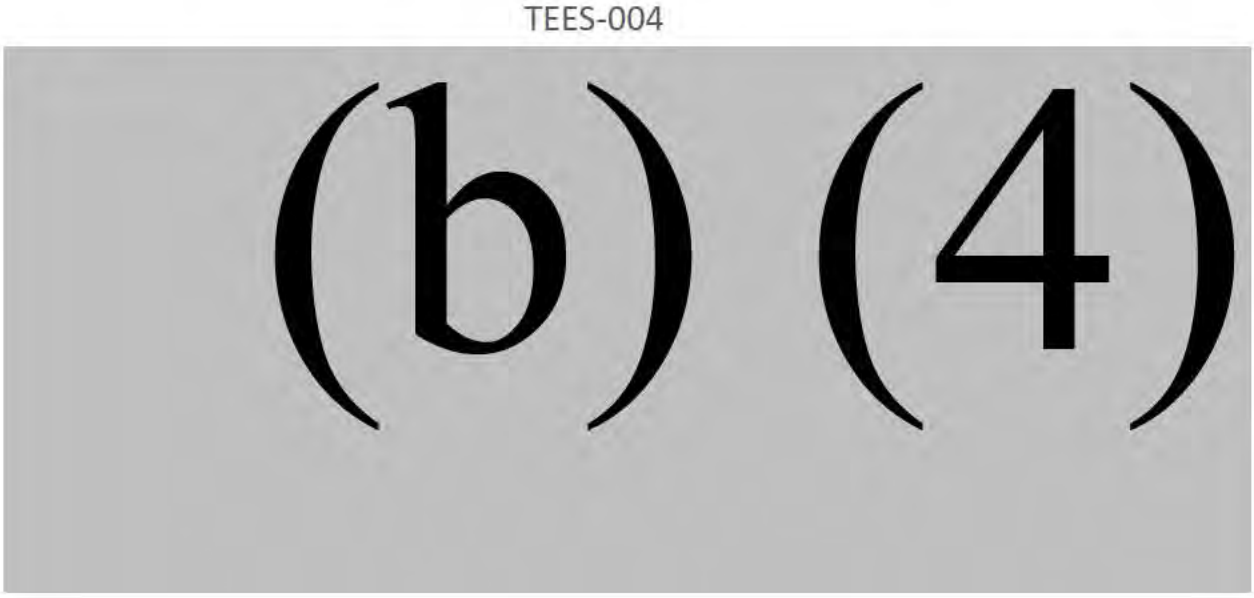


Figure 6 – Run 2

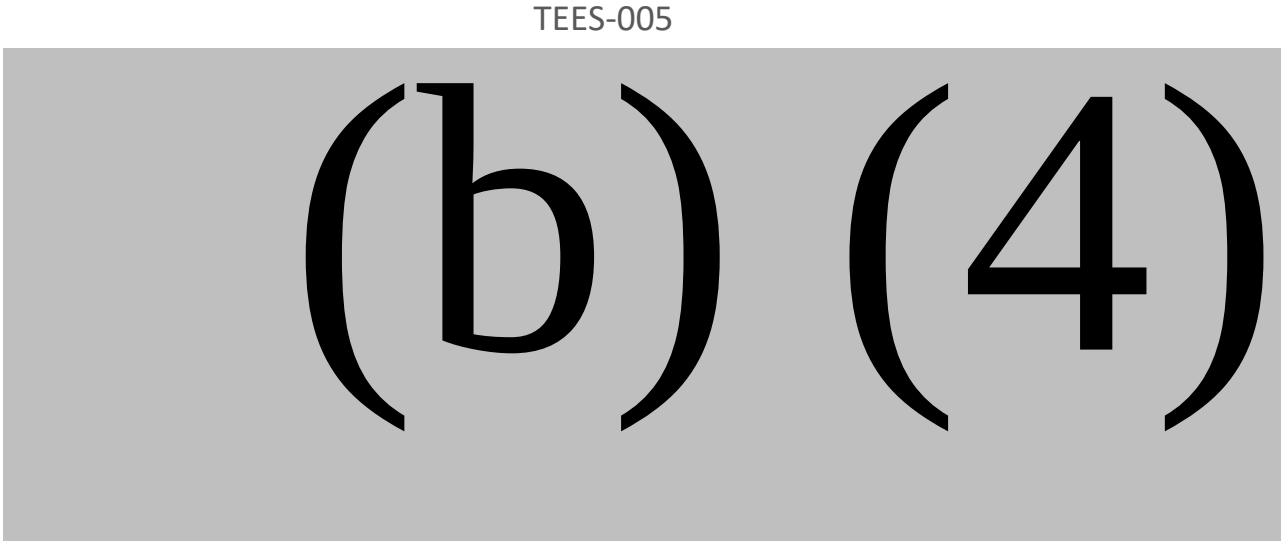
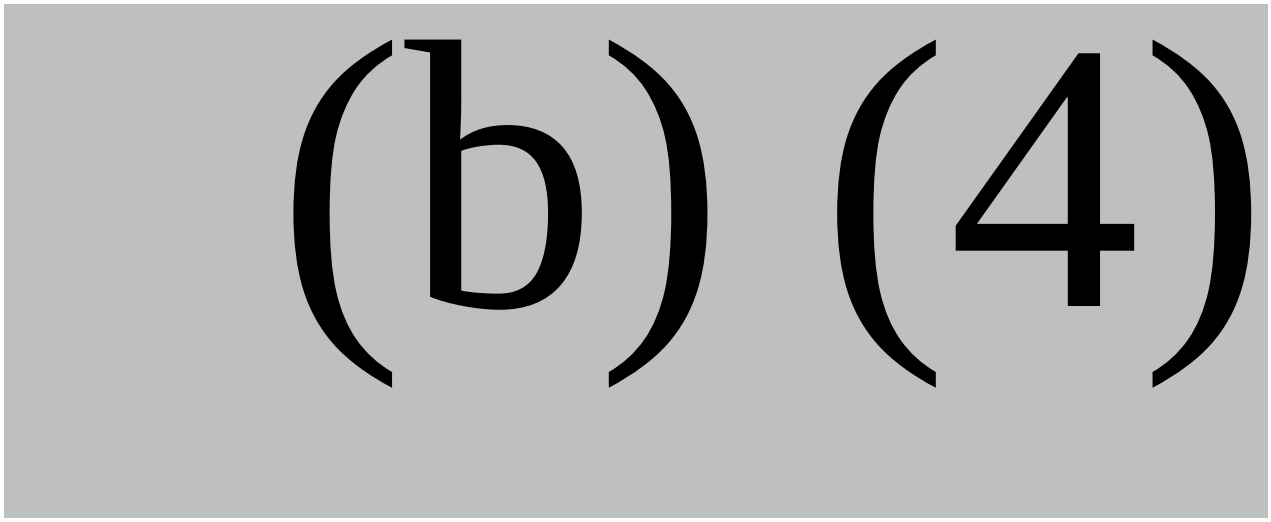


Figure 7 – Run 3



The test data and figure plotting clearly indicates that there is no accumulation of any heavy metals over the course of multiple continuous fermentation runs.

The NAS (2005) MTLs for the protection of the health in fish is 5, 10, 10, 1, and 50 mg/kg in fish feed for arsenic, cadmium, lead, methyl mercury, and nickel, respectively. The feed will contain no more than 18% FeedKind®. Thus, FeedKind® containing no more than 27.8, 55.6, 55.6,

and 277.8 mg/kg arsenic, cadmium, lead, methyl mercury, and nickel, respectively,[57] cannot increase the concentrations of these metals in the feed above their respective MTLs. As noted above, a conservative upper limit specified for chronic exposure to Hg in FeedKind®, based on the NAS (2005) MRL for MeHg for the protection of human health and a compilation of exaggerative exposure assumptions, is 0.01 mg/kg. As shown in Table 11 and Figures 5, 6, and 7, the concentrations of arsenic, cadmium, lead, and nickel in the FeedKind® samples analyzed were well below their respective NAS (2005) MTLs, and the Hg concentrations were well below the MTL for MeHg and the specification for Hg derived above from the NAS (2005) MRL for MeHg for the protection of human health.

Therefore, it can be concluded that the heavy metals do not pose any potential contamination or safety concerns. To ensure future products contain similar levels to the data provided above, Calysta will maintain current testing and monitoring protocols, in addition to adhering to the raw material specifications in Table 6.

2.5 Stability testing

Two separate samples of each of four individual lots of FeedKind® have been stored under controlled conditions for stability testing. Storage will continue for 156 weeks (which is longer than the expected shelf life for FeedKind® (i.e. 52 weeks)) and will generate sufficient data to accurately set a shelf life of FeedKind®. Samples have been chosen at random from different batches of FeedKind® during production runs on March 27, 2017 (TEES004/11), April 11, 2017 (TEES004/29a), and September 21, 2017 (TEES005/28). A single sample from each batch was separated into 20 samples of 500g each. One sample was tested for the 0-week timepoint and the remaining samples (9 each) were placed in temperature and humidity-controlled cabinets at 25°C/60%RH to represent real time testing or 40°C/75%RH to represent accelerated testing. The sample containers used are HDPE, to replicate the polyethylene bulk sacks that may be used at a commercial scale. Holes have been drilled in the lids to allow air into the sample container to represent leakage or absorption at full scale, such that all test conditions represent 'worst-case' conditions. Only real time results are reported here as they are most representative of actual shelf life conditions. Full interim results are available in **Appendix 10.** Sample designations are given in **Table 12**.

Table 12. Samples for Stability Testing

Test Number	Batch	Test Conditions
Stability Test 03	TEES004/29a	(b) (4)
Stability Test 05	TEES004/11	
Stability Test 07	TEES005/28	

[57] For example, feed containing 18% FeedKind® containing 27.8 mg As/kg would have no more than 5 mg As/kg if the arsenic concentration in the other ingredients of the feed, combined, is no more than 5 mg As/kg (i.e. the MTL for As).

* Not heat treated

The testing plan is given in **Table 13**. Proximate testing refers to testing for crude protein, crude fat, ash, moisture and crude fiber.

Table 13. Test Plan

Time	Testing
0 Weeks	Proximate, microbiology, amino acid profile, biogenic amines
4 Weeks	Proximate, microbiology, biogenic amines
8 Weeks	Proximate, microbiology, biogenic amines
12 Weeks	Proximate, microbiology, biogenic amines
26 Weeks	Proximate, microbiology, biogenic amines
39 Weeks	Proximate, microbiology, biogenic amines
52 Weeks	Proximate, microbiology, amino acid profile, biogenic amines
78 Weeks	Proximate, microbiology, biogenic amines
104 Weeks	Proximate, microbiology, amino acid profile, biogenic amines
156 weeks	Proximate, microbiology, amino acid profile, biogenic amines

Proximate and microbiological test results for real time testing through week 52 are available and given in **Table 14**. Full test results including for accelerated testing through week 52 are available in **Appendix 10.** Relevant method descriptions for (b) (4) validated and accredited methods, as well as validation summaries for those methods not (b) (4) accredited, are included in Appendix 8. Yeast and mold methods are not accredited, and summaries are included in Appendix 11.

Table 14. 52 Week Stability Testing Results

Batch TEES004/29a 25°C/60%RH (real time)					
Duration (Weeks)	**Moisture (Max 10.5%)**	**Crude Fat (Min 5%)**	**Crude Protein (Min (b) (4))**	**Crude Fiber (Max 1%)**	**Ash (Max (b) (4)**

0	(b) (4)
4	
8	
12	
26	
39	
52	
78	
104	
156	

Microbiological Analysis

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	(b) (4)			
4				
8				
12				
26				
39				
52				
78				
104				
156				

BatchTEES004/11 25ºC/60%RH

Nutritional Analysis					
Duration (Weeks)	**Moisture (Max 10.5%)**	**Crude Fat (Min 5%)**	**Crude Protein (Min (b) (4))**	**Crude Fiber (Max 1%)**	**Ash (Max (b) (4)**
0	(b) (4)				
4					
8					
12					
26					
39					
52					
78					
104					
156					

Microbiological Analysis				
Test Duration (Weeks)	**TVC (Anaerobic @ 30ºC) cfu/g**	**TVC (Aerobic @ 30ºC) cfu/g**	**Yeasts cfu/g**	**Moulds cfu/g**
0	(b) (4)			
4				
8				
12				
26				
39				
52				
78				

104	(b) (4)
156	

Batch TEES005/28 25ºC/60%RH

Nutritional Analysis

Duration (Weeks)	Moisture (Max 10.5%)	Crude Fat (Min 5%)	Crude Protein (Min (b) (4)	Crude Fiber (Max 1%)	Ash (Max (b) (4)
0	(b) (4)				
4					
8					
12					
26					
52					
78					
104					

Microbiological Analysis

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	(b) (4)			
4				
8				
12				
26				
39[58]				

[58] Data for this sample at this time point is missing.

52[59]	(b) (4)
78	
104	

The initial findings of the shelf life study show FeedKind® to be a stable but hygroscopic product. The data for real time storage when fully open to atmosphere support stability for at least 52 weeks under normal conditions, and accelerated testing confirms that no safety issues are observed with degraded product. The only significant changes observed in the composition of the samples under either real-time or accelerated conditions are the moisture and protein levels, although they remain within specification for the real time aged product. It is also important to note that the sample containers were open to atmosphere under the test conditions such that water absorption from the atmosphere is not unexpected. As the FeedKind® moisture content increases, relatively less of the other components are present in the sample, consistent with the amount of moisture gained diluting the other components. There is no significant change in the protein level when calculated on a dry matter basis. The commercial packaging for FeedKind® will be sealed to help maintain the moisture and protein content within specification.

In an older stability testing performed in 1994 (**Appendix 12**) on product manufactured with the same bacteria and using the same methods, the storage of FeedKind® was monitored for 64 weeks at 22°C and 37°C. A 10 kg sample was divided into nine subsamples with one sample being analyzed immediately. The remaining 8 subsamples were packed in airtight polyethylene bags and stored in an incubator at the indicated temperature (4 each). Bags were removed and analyzed at 4, 16, 32, and 64 weeks for moisture, crude protein, crude fat, free fatty acids, and amino acids (cysteine, methionine, threonine, and lysine). The analyses indicated that the moisture content changed over time (increased at 22°C and decreased at 37°C), but that protein as a percent of dry matter remained steady. Slight decreases in crude fat and increases in free fatty acids were observed and indicate a slow oxidative deterioration of fat over the storage period. Though not all current specifications were tested, these results suggest satisfactory storage stability for the proposed shelf life of 1 year for FeedKind® and that, when properly stored, FeedKind® will not pick up substantial amounts of moisture from the atmosphere. Further, because the more recent testing was performed under circumstances by which the material was open to the atmosphere, we believe that the single out of specification moisture measurement is a result of an overly-aggressive test protocol rather than an indication that, when properly stored, the material is not viable up to (and beyond) 52 weeks.

With regard to the absence of nickel testing, because nickel is not expected to be gained or lost during storage, the adherence to the new specification at the time of production is sufficient to confirm adherence to this specification after long-term storage.

[59] The data for Batch TEES005/28 25ºC/60%RH clearly indicates that the test at 52 weeks for TVC (Aerobic @ 30ºC) cfu/g (700,000 cfu/g) was a spurious result. Tests at 72 and 104 weeks show results in line with the other time points.

2.6 Information on the technical effect of FeedKind®

FeedKind® is a biomass to be used as a protein source for animal feed. FeedKind® is intended for use in the species and at the levels listed in Section 1.4 above.

Part 3 – Dietary exposure

FeedKind® will be included in formulated diets for salmonid species as a replacement for traditional protein sources (*e.g.* soy meal, fish meal, etc.) at inclusion rates of up to 18%. **Table 15** is a comparison of the essential amino acid content of FeedKind® and traditional protein sources commonly found in animal feeds and indicates that FeedKind® is an appropriate replacement for other sources of protein.

Table 15. Essential Amino Acid Composition of Protein Sources for Animal Feed (g/100g dry matter)

Amino Acid	Fish Meal[60]	Soy Meal[61]	FeedKind®[62]
Arginine	4.0	3.43	4.35
Histidine	1.38	1.22	1.5
Isoleucine	2.65	2.1	3.04
Leucine	4.54	3.57	5.22
Lysine	4.78	2.99	3.9
Methionine	1.74	0.68	1.84
Phenylalanine	2.57	2.33	2.9
Threonine	2.83	1.85	2.92
Tryptophan	0.70	0.65	1.1
Tyrosine	2.07	0.40	1.78
Valine	3.00	2.26	3.84

Section 6 includes detailed summaries of well conducted safety studies on salmonids. The No Observed Adverse Effect Levels (NOAELs) from those studies, as expressed as a percent of the diet are:

- Atlantic salmon: 19.3%

[60] Ween O, *et al* 2017. Nutritional and functional properties of fishmeal produced from fresh by-products of cod (Gadus morhua L.) and saithe (Pollachius virens). *Heliyon*. 3(7): e00343. Ween *et al* is used because it is the most complete reference, but the values vary based on type of fish used to generate the fish meal. For example: cod (Ween 2017) vs. pollock in Folador JF *et al*. (2006) Fish meals, fish components, and fish protein hydrolysates as potential ingredients in pet foods. *J Anim Sci*. 84: 2752-2765.

[61] US Soybean Export Council (2015) https://ussec.org/wp-content/uploads/2015/10/US-Soybean-Meal-Information.pdf

[62] Average of three batch analyses in **Table 9.**

- Rainbow trout: 18%

We conservatively utilize the lowest NOAEL of all of the salmonid species tested (18% in rainbow trout) when determining the maximum use level. Exposure to FeedKind® will therefore be no more than 18% of the diet for salmonids.

3.1 Human exposure through consumption of target animals

None of the substances in FeedKind® fed to animals is expected to be transferred, intact, to people consuming the edible products of any of the food-producing animals. The composition of FeedKind® is like that of other common animal feeds, including amino acids, phospholipids, and lipopolysaccharides. Therefore, FeedKind® consumed by the target animals will be digested and converted to biomass and as such there is no expected exposure to FeedKind® for humans *via* consumption of target animals fed FeedKind®.

Part 4 – Self-limiting levels of use

Farmers, aquaculturists, and feed manufacturers using FeedKind® will limit the inclusion of FeedKind® in feed to levels that will not harm or reduce growth rates in the animals being fed. Use will be further limited to 18% in salmonid species, consistent with this GRAS Notice.

Part 5 – Experience based on common use in food before 1958

N/A

Part 6 – Narrative

6.1 Target animal safety summary

Several published studies have evaluated the safety and efficacy of feed formulations containing FeedKind®.[63] These studies typically refer to the test article as "bacterial protein meal" (BPM), which is the biomass product of a bacterial consortium grown on natural gas (methane) as the sole carbon source. The consortium consists of a majority (~90%) of *Methylococcus capsulatus* (Bath) with the remaining 10% consisting of three heterotrophic strains: *Cupriavidus sp.* (previously *Alcaligenes acidovorans* DB3), *Aneurinibacillus danicus* (previously *Bacillus brevis* DB4), and *Brevibacillus agri* (previously *Bacillus firmus* DB5), all of which were isolated from mixed cultures growing on methane.[64]

The data and literature presented in this notification support Calysta's conclusion that use of FeedKind® is safe and GRAS when incorporated at 18% or less of aquaculture feed for salmonid species. This conclusion is corroborated by a number of studies described below, in which salmonids were fed FeedKind® with no adverse effects and no effect on the growth of the animals. This conclusion is also corroborated by ample evidence from the literature and other experimental data derived by Calysta and others.

Regarding the use of FeedKind® in aquaculture, Calysta views Atlantic salmon and rainbow trout as representative models for all salmonid species, including Arctic char and Coho salmon, for example.[65] Among the important considerations for defining representative species include the life cycle, diet consumed in nature, physiology and metabolism, available background information, and commercial relevance.

Calysta believes that the data from investigations of the common salmonids Oncorhynchus mykiss (rainbow trout) and Salmo salar (Atlantic salmon) are sufficient to support the broader use of the notified substance for all aquaculture feed for salmonid species, with these two test species fulfilling all of the criteria required for covering all of the species of the phylogenetic family Salmonidae, including the requirement that test subjects be well-studied, sensitive to testing, and commercially-relevant.

Salmonid test species generally serve as good surrogates in nutritional studies because species in this phylogenetic family are characteristically sensitive to allergenic substances added to their diets. Gastrointestinal inflammation (gastroenteritis) is a well characterized effect observed in salmonids fed diets containing terrestrial proteins such as soy protein. Salmonids (*i.e.* salmon and trout) represent a substantial fraction of the total commercial value for the industry (Mente et al. 2006; Glencross et al. 2007; Gjedrem et al. 2012; Ababouch et al. 2016).

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[63] The bacteria used for the test articles in the animal studies and for the product manufactured today are the same strains and come from a culture bank. Aside from a slight widening of tolerances for the pH and temperature of the fermenter, further the fermentation parameters have not been changed. We may therefore conclude that the finished product is the same.

[64] As described in Section 2.1, BPM and FeedKind® are identical. The BPM nomenclature is used as a vestige of the nomenclature used in some of the animal studies.

[65] A similar approach was taken in AGRN 26 for application to all finfish species on the basis of studies in several species.

For these reasons, Calysta believes that the data and information presented on rainbow trout and Atlantic salmon in this notification are sufficient to support the finding that the notified substance is Generally Recognized as Safe for use in any aquaculture feed for salmonid species, when incorporated at 18% or less of the feed.

6.1.1 Safety of the microorganisms[66]

A detailed search of the public literature did not find any reports of pathogenicity, infections, or toxin production by any of the members of the consortia utilized to produce FeedKind®. Both old and new taxons were searched. Searches return hits for various *Bacillus* species (*i.e. anthracis* and *cereus*) though there are no organisms currently classified as "*Bacillus*" in FeedKind® and none of the hits for *Bacillus* species implicates the organisms in FeedKind® as potential pathogens. A variety of *Cupriavidus* species have been reported to cause opportunistic infections in humans. *C. metallidurans*[67] and *C. gilardii*[68] have been associated with sepsis in elderly patients with other underlying pathology (diabetes, etc.). *C. pauculus* appears to be associated with the most cases of human disease, with more than 30 cases reported in the literature.[69] While most cases are reported in the very young or very old with or without underlying disease, several appear to be associated with otherwise apparently healthy patients. The effects of *C. pauculus* infection appear to be more severe than those seen with other *Cupriavidus* species with *C. pauculus* being associated with cases of meningitis, respiratory tract infections, septicemia, and at least 3 deaths. As with the literature search hits for *Bacillus*, the species of *Cupriavidus* for which the literature indicates potential pathogenicity are not the same as is used in FeedKind®. There is a single report of a urinary tract infection (UTI) caused by a *Brevibacillus* organism that was later determined to be *Brevibacillus agri* through 16s sequencing. However, additional characterization of the strain which caused the infection indicated that the *B. agri* strain in question had acquired genes related to pathogenicity, likely through horizontal transfer, including hemagglutination and serum resistance that are not typically present in *Brevibacillus agri*.[70] There were no reports of infections caused by any

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66 The data supporting the safety of the microorganisms can be found summarized in the April 28, 1995 Scientific Committee for Animal Nutrition report, found in **Appendix 13**. The accepted nomenclature for these bacteria has changed based on modern molecular (*i.e.* sequencing) techniques. However, the bacteria used to produce FeedKind® has not changed relative to those used to conduct the animal studies. Further, a recent literature search to confirm that these bacteria are not known to be human or animal pathogens did not yield any new results that might change the conclusion reached in 1995.

67 Langevin S, Vincelette J, Bekal S, and Gaudreau C. (2011) First case of invasive human infection caused by *Cupriavidus metallidurans*. *J. Clin. Microbiol.* 49 (2): 744-745.

68 Kobayasi T, Nakamura I, Fujita H, Tsukimori A, Sato A, Fukushima S, Ohkusu K, Matsumoto T. (2016) First case report of infection due to *Cupriavidus gilardii* in a patient without immunodeficiency: a case report. *BMC Infectious Diseases*. 16: 493; Zhang Z, Deng W, Wang S, XuL, Yan L, Liao P. (2017) First case report of infection caused by *Cupriavidus gilardii* in a non-immunocompromised Chinese patient. *IDCases*. 10:127-129.

69 Yahya R, and Mushannen A. (2019) *Cupriavidus pauculus* as an emerging pathogen: a mini-review of reported incidents associated with its infection. *EC Pulmonology and Respiratory Medicine* 8(9): 633-638.

70 Suneeva SC, *et al* (2014) Transformation of *Brevibacillus,* a soil microbe to an uropathogen with hemagglutination trait. *World J Microbiol Biotechnol*. 30 (6) 1837-1844.

Methylococcus or *Aneurinibacillus*. The pathogenicity of the heterotrophic strains is further addressed by the rodent studies described below.

Methanotrophic bacteria are not known to be human or animal pathogens. They require single-carbon energy sources (*e.g.* methane or methanol) for growth and, thus, are not believed to pose any danger to humans or animals. *M. capsulatus* (Bath) has a high optimal growth temperature (45°C), which is substantially greater than the normal body temperatures of mammalian species,[71] providing another indication that it is unlikely to become a human or animal pathogen.

Cupriavidus sp., *Aneurinibacillus danicus,* and *Brevibacillus agri* showed no signs of pathogenicity in mice injected with 9.5 x 10^9, 5.3 x 10^9, and 2.9 x10^9 viable cells/kg body weight, respectively, which were the highest doses tested. *Cupriavidus sp.* has a high optimal growth temperature (44°C) similar to that of *M. capsulatus* and is restricted to utilization of carbon sources with chain lengths from 2-4 carbons. The highest dose of both *Brevibacillus agri* and *Aneurinibacillus danicus* did induce signs of acute toxicity. However, these effects were transient (lasting a few hours to 3 days after exposure) and were consistent with effects expected following injection of large amounts of organic material. The pathogenicity studies indicate that none of the constituent organisms used in the FeedKind® production process is pathogenic to animals. The studies are summarized below:[72]

Cupriavidus sp. was administered intravenously to 5 male and 5 female mice at doses of 0, 6.3 x 10^5, 16.7 x 10^7, and 9.5 x 10 ^{9} viable cells/kg bw. Animals were observed for 14 days and then killed and subjected to pathological examination. There were no clinical signs of reaction to the treatment during the observation period or upon necropsy. As a Gram-negative organism, *Cupariavidus sp.* contains endotoxins associated with the cell wall. However, as there are no adverse effects indicated by this assay, they clearly pose no safety concerns.

Aneurinibacillus danicus was administered intravenously to 5 male and 5 female mice at doses of 0, 3.0 x 10^5, 2.9 x 10^7, or 5.3 x 10^9 viable cells/kg bw. Animals were observed for 14 days and then killed and subjected to pathological examination. Clinical signs including piloerection and depression were seen in all dosed groups but were transient. Signs lasted for 3 hours, 1 day, and 3 days respective to increasing dose. Males generally exhibited signs for longer than females, however one female in the highest dose group died approximately 1 hour after dosing. No clinical signs of pathogenicity were observed during the study or during necropsy.

Brevibacillus agri was administered intravenously to 5 male and female mice at doses of 0, 9.4 x 10^5, 2.3 x 10^7, or 2.9 x 10^9 viable cells/kg bw. Animals were observed for 14 days and then killed and subjected to pathological examination. Similar to *Aneurinibacillus danicus*, clinical signs including piloerection and depression were seen in the medium and high dose groups but were transient. Signs lasted for 1 day or 3 days respective to increasing dose. Males generally exhibited signs for longer than females, however females exhibited a greater degree of depression

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71 For example, normal human body temperatures range from 36°C to 37°C.

(b) (4)

[redacted]

immediately after dosing. No clinical signs of pathogenicity were observed during the study or during necropsy.

6.1.2 Salmonid species

One freshwater and two saltwater feeding studies were performed in Atlantic salmon at the Institute for Aquaculture Research in Norway and published in the peer-reviewed journal Aquaculture from 2004 through 2006. These studies are summarized below.

In the freshwater study, Storebakken *et al.* (2004) fed groups of Atlantic salmon (*Salmon salar*) (n=600/group; 3 groups/diet; average initial body weight 0.2 g/fry) 0%, 5%, 10%, 19.3%, or 37% BPM equivalent to FeedKind® in the diet for 364 days, starting with the first feeding at the fry stage of the life cycle. The BPM added to the feed replaced an equivalent amount of high-quality fish meal in the feed in each group of exposed animals.[73]

After the first 112 days of exposure, the salmon fed 5% BPM exhibited the greatest average body weight (bw = 3.69 ± 0.07 g) and specific growth rate (SGR =2.64 ± 0.02), both of which were statistically significantly greater than the corresponding control values (i.e. bw = 3.44 ± 0.22 g; SGR = 2.57 ± 0.06).[74] As well, the average bw and SGR were statistically-significantly elevated in the fish fed 5% BPM compared to fish fed BPM at any of the other inclusion levels.[75] Both of these parameters were statistically significantly reduced in the fish fed 37% BPM (bw = 2.63 ± 0.08; SGR = 2.33 ± 0.03), compared with controls. However, the SGRs of Atlantic salmon fed 5%, 10% or 19.3% BPM for 112 days were not statistically-significantly different from the SGR of the fish fed the control diet.

From day 113 to 253 of the exposure period, the SGRs were statistically significantly lower in fish fed 19.3% BPM (SGR = 0.60 ± 0.05) and 37% BPM (SGR = 0.51 ± 0.05), compared with controls (SGR = 0.74 ± 0.03). By comparison, the SGRs calculated for fish exposed to 5% or 10% BPM were not statistically different from each other or from the control values.

However, from day 254 to 364, the SGRs calculated for fish fed 5%, 10%, or 19.3% BPM were not statistically significantly different from controls and the final average body weights were statistically significantly elevated compared with controls (e.g., bw = 46.2 ± 1.6 g for fish fed 19.3% BPM compared with 38.2 ± 3.0 g for controls). The final average bw and SGR were statistically significantly reduced only for fish fed 37% BPM (bw = 28.0 ± 2.3 g; SGR = 0.82 ± 0.06), compared with controls (bw = 38.2 ± 3.0 g; SGR = 0.97 ± 0.03). Likewise, cumulative survival rate for the day 113 to 364 exposure period was statistically significantly reduced only for fish fed 37% BPM (98.0 ± 0.4%) compared with the controls (99.6 ± 0.00%).

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[73] Storebakken T, *et al.* (2004) Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo salar*, in freshwater. *Aquaculture*. 241: 413-425.

[74] Bulk weight of all fish in each tank was measured every 28 days, mean weight per fish (w) was calculated from the bulk weight and the number of fish remaining in the tank, and specific growth rate (SGR) was calculated; SGR = $100(e^{x}-1)$, where x = (ln (w_{final} – ln w_{start}) – ln w_{start}) ÷ days fed.

[75] SGR of fish fed 5% BPM was 4.5%, 6.8% and 12% greater than the SGR for fish fed BPM at 10%, 19.3%, and 37% in the diet, respectively, for 112 days.

In sum, bw and SGR values were indistinguishable or improved in salmon receiving feed containing 5%, 10% or 19.3% BPM in the diet, compared with controls, over the first 112 days of the exposure period in the study conducted by Storebakken et a. (2004). These values were statistically significantly reduced during the exposure period extending from day 113 to day 252 but recovered to be indistinguishable from controls during the final period extending from day 254 to day 364. The reduced bw and SGR measurements observed over the 113-day to 252-day period are attributable to two factors related to the experimental protocol of this study, including:[76]

- Infrequent size grading of the fish, which was done to keep undersized salmon in the population and, thus, increase the probability of detecting any long-term histopathological effects of BPM on the salmon
- Pellet sizes too large for fish at earlier life stages, which should have been changed to correspond with the increasing size of the fish, but the pellet sizes used were selected to minimize the potential for feed batch variations to affect the results

Generally, there is substantial body size variability in farmed fish of the same age. Periodically grading and sorting the fish based on body size enables feeding the fish food pellet sizes that are appropriate for their body size, which improves feed conversion efficiency among other beneficial effects of this practice. In comparison, Storebakken et al. (2004) graded and sorted the fish only on day 253 of the exposure period. During the first 112 days of the exposure period, Storebakken et al. (2004) fed the fish experimental diets that were prepared by cold-pelleting the feed formulation through a 5-mm die on a laboratory mill, and then crumbling the pellets with a coffee grinder and sieving the particles to produce the appropriate pellet sizes for fish. From day 113 to day 364 of the exposure period, the fish received the diets that were cold-pelleted through a 3-mm die, and these pellets were not crumbled before feeding to the fish. Thus, the reduced average bw and SGR measurements calculated for the fish during day 113 to day 252 of the exposure period are attributable to feeding the fish pellet sizes that were too large for many, if not most, of the fish, especially during the early days of this period. The complete recovery of the fish during the final day 252 to day 354 exposure period lend considerable weight to this conclusion. Gut-to-body-weight ratio and whole-body fat tended to increase with increasing dietary BPM concentration, and gut-to-body-weight ratios and liver-to-body weight ratios were slightly, but statistically-significantly, elevated in the fish fed 37% BPM in the diet for 364 days. However, histological evaluations revealed no evidence of disease and no systematic differences in the tissues of the fish exposed to 5% or 37% BPM for 252 days, except for reduced hepatocellular vacuolization in the fish fed 37% BPM.[77]

Storebakken et al. (2004) assessed nutrient digestibility indirectly by feeding salmon (n=50/group, 3 groups/concentration; mean body weight 60 g) diets containing 0%, 5%, 10%, 19.3%, or 37% BPM equivalent to FeedKind® for 14 days. Cr_2O_3 was added to the diets as a marker before pelleting the formulations. As in the main study, the BPM added to the feed replaced an equivalent amount of fish meal in the feed of each group of exposed animals. The feces were collected by manual stripping after the exposure period. Total concentrations of nitrogenous

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[76] Storebakken T, *et al.* (2004) Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo salar*, in freshwater. *Aquaculture*. 241: 413-425.

[77] Fish (n=10/2 of 3 replicate tanks/diet) exposed to 5% or 37% BPM for 252 days were sampled for histological examination to include poorly growing fish that were discarded during size grading.

substances (including proteins and nucleic acids) and fat were measured in feces, and apparent digestibility coefficients (ADCs) were calculated for nitrogenous compounds (i.e. "nitrogen digestibility") from the nutrient-to-marker ratios of the diet and the feces.

The ADCs for nitrogen digestibility were statistically significantly lower in the salmon receiving BPM in the diet, compared with controls. The ADCs for nitrogen digestibility were 89.9%, 88.1%, 88.3%, 86.7%, and 84.2% for salmon receiving 0%, 5%, 10%, 19.3%, and 37% BPM in the diet, respectively. Thus, the ADCs were lower than the control value by 1.8%, 1.6%, 3.2%, and 5.7% in the salmon fed diets containing 5%, 10% or 19.3%, and 37% FeedKind®, respectively. The authors were able to fit the ADCs for nitrogen reasonably well to a straight line (r=0.95) after omitting the ADC for the salmon exposed to 5% BPM. They estimated the nitrogen digestibility to be 78.4% from this curve, assuming that 100% replacement of fish meal with BPM in the diet.[78] Although this estimate was not corrected for differences in the content of nitrogenous non-protein substances (i.e. mainly nucleic acids, including 2.2% DNA and 7.3% RNA in BPM), the authors indicated that this value represents the digestibility of crude protein from BPM. They noted that this result is consistent with the 81.9% total nitrogen digestibility reported by Skrede et al. (1998), who fed salmon BPM as the sole source of protein.[79]

The ADCs for fat digestibility were approximately 96.4%, 96.2%, 95.8%, and 95% for salmon receiving 0%, 5%, 10%, 19.3% and 37% BPM in the diet, respectively. Although there appears to be a downward trend in the ADC with increasing BPM, only the ADC for fat digestibility for salmon fed 37% BPM was statistically significantly lower than the control value. The authors noted that Storebakken et al. (1998) and others found no effect on the ADC for lipid digestibility in salmon fed a diet in which BPM replaced fish meal. They noted that their results, particularly in the animals fed the diet containing 37% BPM, may be attributable to the presence of relatively high levels of non-starch polysaccharides, which are known to reduce the absorption of fats in the diet.

In a saltwater experiment, Berge *et al.* (2005) fed groups of Atlantic salmon (n=1000/pen; 2 pens/diet; average initial body weight = 1.39 kg) 0%, 10%, or 20% BPM equivalent to FeedKind® in the diet (equivalent to 0%, 17.2% and 33.1% dietary nitrogen, respectively) for 5 months.[80] As in the freshwater study, the BPM added to the feed replaced an equivalent amount of high-quality fish meal in the feed.[81]

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[78] ADC for nitrogen = 89.76-(0.113 x 100) = 78.46%;

[79] Skrede A, Berge GM, Storebakken T, Herstad O, Aarstad KG, Sundstol F (1998). Digestibility of bacterial protein grown on natural gas in mink, pigs, chicken, and Atlantic salmon. Animal Feed Sci. Technol. 76(1-2): 103-116.

[80] Berge GM, *et al.* (2005) Bacterial protein grown on natural gas as protein source in diets for Atlantic salmon, *Salmo salar*, in saltwater. *Aquaculture*. 244: 253-240.

[81] Daily feed intake was quantified for each pen as the difference between the ration fed and the collected excess feed corrected for leaching in sea water. Salmon were counted and weighed individually at the start of the experiment and 2, 4, and 5 months thereafter, and salmon were sampled (n=10/pen at start and 5/pen thereafter) for body composition analyses. Salmon (n=10/pen) were sampled for fat-content estimation via computerized tomography. Digestibility was estimated based on the results of analyses of feces collected at 4 months and fish collected (minimum n=60 fish samples pooled/pen). Histological examination and hematocrit measurement were performed on fish collected after 5 months of exposure (n=5/pen).

During the initial 3 weeks of the exposure period, mortalities were frequent regardless of the BPM content of the feed (i.e., 13.9 ± 1.3%, 17.3 ± 3.6% and 9.0 ± 0.1% at 0%, 10%, and 20% BPM in the diet, respectively) but were not statistically significant among the control and BPM-exposed groups. Mortality was low throughout the rest of the study. No statistically significant effects were found on mean body weights, growth rates, feed intake, or feed conversion ratio (FCR) measured after 2 months and after 5 months of exposure and analyzed by analysis of variance (ANOVA).

However, Berge et al. (2005) noted that body weights and thermal growth coefficients were inversely correlated with dietary BPM concentration at 2 months and 5 months when the data were analyzed by linear regression.[82] However, Aas et al. (2006a) re-analyzed the data reported by Berge et al. (2005) and reported that there were no statistically significant differences in the mean body weights of the fish fed 10% or 20% BPM in the diet for 2 months or 5 months, compared with controls.[83] Aas et al. (2006a) found that the only statistically-significant difference in body weights was between the fish fed 10% BPM and those fed 20% BPM for 2 months, and there was no statistically significant difference in body weights between the fish fed 10% BPM and the fish fed 20% BPM at the end of the 5-month exposure period.

Carcass and visceral dry matter and visceral fat and dry matter contents were also statistically-significantly inversely correlated with dietary BPM levels, but there were no detectable effects on dressed-out carcass, liver, or intestine weight-to-body-weight ratios. There appeared to be trends of decreasing digestibility of nitrogen, fat, and energy with increasing BPM concentration in the diet, but none of the trends were statistically significant in ANOVA or regression analysis of the data. There were no statistically significant differences in whole-body nitrogen retention among any of the animal groups.

Histological examinations indicated that the mucosa of the distal intestines was generally normal, including absorptive vacuoles in the enterocytes of the intestinal folds and moderate amounts of leucocytes infiltrating the mucosa and submucosa across the animal groups.

Only one fish, a male fed 10% BPM in the diet, exhibited severely inflamed intestinal mucosa, heavy leucocyte infiltration of the mucosa, and no absorptive vacuoles, without the reduction in mucosal-fold height reported to be induced by soybean meal.[84] However, the body

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[82] At 5 months, mean body weights were 3889 ± 32, 3776 ± 101, 3649 ± 63 g/fish and thermal growth coefficients were 2.89 ± 0.03, 2.79 ± 0.09, and 2.67 ± 0.00 for fish exposed to 0%, 10%, and 20% BPM in the diet, respectively.

[83] Aas TS, et al. (2006a) Improved growth and nutrient utilization in Atlantic salmon (Salmo salar) fed diets containing a bacterial protein meal. Aquaculture. 259: 365-376.

[84] A related study showed that increasing dietary concentrations of BPM combined with 200 g/kg solvent-extracted soybean meal attenuated the typical soybean-meal-induced disturbances observed in the distal intestines of Atlantic salmon; the levels of inflammatory regulators $CD8\alpha^+$ T lymphocytes and MHC II-reactive cells observed in the intestinal tissues of the soya-extract-expose animals were normalized by sufficient inclusion of BPM in the diet, the regulatory mechanisms for these effects are not yet known. *See* Romarheim et al. (2012 online). Prevention of soya-induced enteritis in Atlantic salmon (Salmo salar) by bacteria grown on natural gas is dose dependent and related to epithelial MHC II reactivity and $CD8\alpha^+$ intraepithelial lymphocytes. Brit. J. Nutr. March 2013: 1-9.

weight and length of this fish was close to the tank means, and the fish had no external signs of disease and had normal hematocrit measurements after 364 days of exposure to BPM. In general, none of the fish examined exhibited signs of allergic reaction to BPM in the distal intestinal mucosa, even those exposed to 20% BPM in the diet for 364 days, and all of the fish had hematocrit measurements within the normal range.

Likewise, evaluation of carotenoid concentration and sensory characteristics of the flesh showed no differences between controls and the fish fed BPM at any concentration.

Berge et al. (2005) used the same indirect method as Storebakken et al. (2004) to assess the digestibility of nitrogen, fat, and energy, except that Y_2O_3 was used as the marker, rather than Cr_2O_3. Salmon (n=60/pen minimum) were fed diets containing 0%, 10%, or 20% BPM for 4 months, after which fecal samples were collected by manual stripping and the samples from each pen were pooled, homogenized and analyzed. The nutrient digestibility estimated for salmon raised in inner pens were statistically significantly greater than the corresponding values estimated for salmon raised in the outer pens, which is consistent with the observation that the salmon in the inner pens were less stressed, because of reduced exposure to the open sea, than the salmon in the outer pens. In any case, the were no statistically significant differences between the exposed salmon and the controls in the ADC measurements for nitrogen (ADC = 86.8%, 84.9%, and 83.1% for 0%, 10%, and 20% BPM, respectively), fat (ADC = 88.5%, 86.0%, and 84.0% for 0%, 10%, and 20% BPM, respectively), and energy (ADC = 84.4%, 82.0%, and 78.6% for 0%, 10%, and 20% BPM, respectively), and no statistically significant concentration-response relationship was evident by regression analysis in this study.

Berge et al. (2005) found no statistically significant differences in nitrogen retention in the salmon receiving BPM in the diet, compared with controls, or any evidence of a concentration response trend in this parameter.

Like Storebakken et al (2004), Berge et al. (2005), noted that the mean ADCs for nitrogen digestibility were lower in salmon fed BPM than in the controls (e.g., 83.1% in salmon fed 20% BPM vs. 86.8% in salmon fed at 0% BPM in the diet). The ADCs for nitrogen digestibility reported by Berge et al. (2005) for salmon receiving diets containing 0%, 10% and 20% BPM are comparable to those reported by Storebakken et al. (2004) for salmon, which ranged from 84.2% in salmon fed 37% BPM to 89.9% in salmon fed at 0% BPM in the diet. Like Storebakken et al. (2004), Berge et al (2005) noted that their results illustrate a tendency for poorer digestibility of the crude protein of BPM. However, Berge et al. (2005) acknowledged that a tendency for reduced nitrogen digestibility of BPM, compared with high quality fish meal, can be attributed to the presence in BPM of bacterial cell walls and membranes that are resistant to enzymatic digestion.

In another saltwater experiment, Aas *et al.* (2006a) fed Atlantic salmon (n=18/group; 3 groups/diet; average initial body weight 170 g) 0%, 4.5%, 9%, 18%, or 36% BPM equivalent to FeedKind® in the diet for 48 days.[85] In a parallel digestibility study, salmon (n=3/group; initial body weight 494 g) were fed 0%, 18%, or 36% BPM in the diet for 15 days. There were no mortality or health problems observed in any of the fish exposed to BPM in the diet for up to 48 days.

[85] Aas TS, *et al.*(2006a) Improved growth and nutrient utilization in Atlantic salmon (*Salmo salar*) fed diets containing a bacterial protein meal. *Aquaculture*. 259: 365-376.

The SGRs of the fish fed 18% or 36% BPM were statistically-significantly greater than the rates of the controls or the fish fed 4.5% BPM.[86] BPM did not affect feed intake.

Increased dietary BPM levels were also associated with reduced branchial[87] and/or renal nitrogen and energy losses and energy spent on activity and maintenance.[88] The liver-to-body-weight and viscera-to-body weight ratios were statistically-significantly lower in the salmon fed 4.5% and 4.5% or 9% BPM, respectively, compared to the controls and to the fish fed higher concentrations of BPM in the diet.[89]

The copper concentrations were greater in the viscera of fish fed 36% BPM,[90] but there were no adverse effects of copper on growth or survival and no discernable differences in copper or phosphorous concentrations in the liver or carcass, dry-matter, fat, nitrogen, ash, or energy contents of the liver, viscera, or carcass, or amino acid content of the whole body. The authors attributed the elevated visceral concentration of copper levels measured in the fish fed 36% BPM to the supplementary copper added to all of the diets in this study (5 mg/kg), as well as to the greater copper content of the BPM compared to that of the fish meal used.[91]

Aas et al. (2006a) assessed nutrient digestibility by feeding salmon (20.4 kg biomass/group, 3 groups/concentration; mean body weight 494 g) diets containing 0%, 18%, or 36% BPM equivalent to FeedKind® for 14 days. Y_2O_3 served as the marker. Like Berge et al. (2005), Aas et al. (2006) calculated ADCs for nitrogen, lipid, and energy, as well as nitrogen retention. In addition, Aas et al. (2006) calculated ADCs for individual amino acids and for the sum of amino acids.

The ADCs calculated for nitrogen digestibility were consistent with those reported by Storebakken et al. (2004) including lower values in the exposed animals compared with controls and a downward trend with increasing BPM concentration in the diet (ADC for nitrogen = 87.8%, 86.6%, and 84.8% for 0%, 18%, and 36% BPM, respectively). In addition, Aas et al. (2006) found comparable trends in the ADCs of individual amino acids, as well as in the ADCs for the sum of amino acids. However, the ADCs for the sum of amino acids (ADC for sum of amino acids = 90.7%, 89.3%%, and 87.6% for 0%, 18%, and 36% BPM, respectively) and ADCs for individual amino acids were greater than the corresponding ADCs for nitrogen digestibility. These results showed that using total nitrogen ADCs to represent the ADCs for protein or amino acids results in the underestimation of these values for BPM. The authors suggested that this effect may be

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86 For example, body weights measured on day 52 averaged 33 ± 12.3, 327 ± 10.7 and 360 ± 3.2 g in fish exposed to 0%, 4.5%, and 36% BPM for 48 days, respectively.

87 Branchial means of or related to the gills.

88 The authors concluded that the reduction in the energy used for activity and maintenance per kg body growth is attributable to the greater growth rates at the highest dietary BPM concentrations tested.

89 For example, the liver-to-body ratios were 1.33 ± 0.04, 1.20 ± 0.02, and 1.38 ± 0.03 in fish fed 0%, 4.5%, and 36% BPM, respectively; the corresponding viscera-to-body-weight ratios were 7.37 ± 0.13, 6.91 ± 0.16, and 7.52 ± 0.09, respectively.

90 Mean [Cu] = 0.1 ± 0.1 mg/kg and 0.2 ± 0.1 mg/kg in viscera of fish exposed to 0% and 36% dietary BPM, respectively.

91 Aas et al. (2006a) reported the copper concentration to be 87.9 ppm in the BPM tested; the copper concentrations in the test diets containing 0%, 4.5%, 9%, 18%, and 36% BPM were 9.6 ppm, 14.4 ppm, 14.9 ppm, 20.4 ppm and 35.6 ppm, respectively.

attributable to the relatively indigestible cell walls in BPM, which are not present in the high-quality fish meal used in these studies.

Furthermore, Aas et al. (2006a) found that ingested and digested nitrogen retention and energy retention and ingested lipid retention were statistically significantly elevated in the salmon fed diets containing 18% or 36% BPM, compared with controls. The authors attributed the absence of adverse effects on mortality rates, growth rates and other indices of health in salmon exposed to up to 36% BPM to the improved utilization of feed containing BPM.

In addition to the published studies in Atlantic salmon summarized above, there is a published study in another species of the Salmonidae (salmonid) family, namely the rainbow trout. In the rainbow trout (*Oncorhynchus mykiss*) experiment, Aas *et al.* (2006b) fed triplicate groups of the fish (n=11/group; initial average body weight = 361 g) 0%, 9%, 18%, or 27% BPM equivalent to FeedKind® or 9% BPM autolysate[92] in the diet for 71 days. The BPM or BPM autolysate replaced the equivalent levels of fish meal and starch of the base diet.[93]

One fish in the group receiving 27% BPM in the diet died. However, there were no statistically significant differences after the 71-day exposure period across the groups in mean body weights, SGRs, feed intake, or FERs, or liver- or viscera-to-body-weight ratios evaluated by ANOVA. Regression analysis suggested increasing liver-to-body-weight ratio with increasing dietary BPM content (p=0.044). However, the relationship between liver-to-body-weight ratio and dietary BPM content did not appear to be linear (r^2=0.35), indicating that this result may be a statistical artifact.

There were no statistically significant differences in nitrogen, crude-lipid, dry-matter, ash, or energy levels in the liver, carcass or viscera across the groups, except for a slightly elevated ash content of the liver of the animals exposed to 27% BPM in the diet. There were no statistically significant differences in the mineral contents of the liver, including copper, in the BPM-exposed animals compared with the controls, and no effects on whole-body amino-acid composition, based on ANOVA. Linear-regression analysis indicated increasing whole-body histidine and decreasing whole-body methionine concentrations with increasing dietary BPM concentration.[94]

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92 Autolysis, aka self-digestion, is the destruction of cells through the action of the enzymes of the cells. The authors provided no details about the production of the BPM autolysate used in the study, except to note that the BPM autolysate and BPM represented two different batches of bacterial biomass, which helps to explain some of the differences in the compositions of the two products tested.

93 Aas TS, *et al.*(2006b). Effects of diets containing a bacterial protein meal on growth and feed utilization in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*. 261: 357-368.

94 For example, whole-body histidine concentrations were 2.79% ± 0.06% and 2.94% ± 0.05% in fish fed 0% and 27% BPM, respectively; the corresponding values for methionine were 3.30% ± 0.03% and 3.23% ± 0.01%, respectively.

Uric acid levels in plasma were also elevated in trout exposed to 27% BPM, compared to controls and trout exposed to 18% BPM in the diet.[95] However, there were no exposure-related effects on urea levels measured in plasma, liver or muscle.[96]

The ADCs for copper were lower than control values and for phosphorus greater than control values for all groups exposed to BPM, and these differences were statistically significant.[97]

There were no statistically significant differences in retention of digested lipid, energy, nitrogen or amino acids among the groups. No exposure-related effects were found in the ANOVA analysis of nitrogen-budget parameters, except for elevated fecal-nitrogen loss. However, regression analysis indicated increasing nitrogen intake per kg body growth with increasing dietary BPM concentration. Fecal energy loss also increased with increasing dietary BPM content above 9%, and the energy used for activity and maintenance[98] was greater in trout receiving 27% BPM and heat increment[99] was lower in trout receiving 9% autolyzed BPM compared with the corresponding values for controls and fish exposed other concentrations of BPM. The energy spent on total heat increased statistically significantly with increasing dietary BPM content, based on regression analysis of the data.

No diet-related morphological changes were observed in the digestive tract, and differences in degree of vacuolization of the epithelial cells of the villous folds of the pyloric caeca were observed in nearly all fish, regardless of the presence or absence of BPM in the diet.

Overall, there were no significant differences in the results obtained with autolyzed BPM compared with BPM.

Aas et al. (2006b) assessed nutrient digestibility by analyzing feces collected during weeks 6 to 9 of the 10-week exposure period. Y_2O_3 (0.1 g/kg) in the diets served as the marker. As in the salmon studies, the ADCs calculated for nitrogen, energy, and sum of the amino acids, exhibited downward trends with increasing dietary BPM concentrations (e.g., ADC for nitrogen = 95.5%, 94.7%, 94.0%, and 90.7% for 0%, 9%, 18%, and 27% dietary BPM, respectively; ADC for sum of amino acids = 96.8%, 96.3%, 96.2%, and 92.4% for 0%, 9%, 18%, and 27% dietary BPM,

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95 Plasma uric acid concentrations were 29.04 ± 3.15, 29.56 ± 2.16, and 43.68 ± 4.47 µmol/l in fish receiving 0%, 18%, and 27% BPM in the diet, respectively.

96 The authors noted a previous study in Atlantic salmon fed high dietary levels of BPM that found elevated urate oxidase activity in the liver and urea concentrations in the plasma, liver, and muscle without statistically-significant elevations in plasma uric acid levels, indicating that uric acid is less stable in trout than in Atlantic salmon; see Anderson *et al*. (2006). Purine-induced expression of urate oxidase and enzyme activity in Atlantic salmon (*Salmo salar*); Cloning of urate oxidase in liver cDNA from three teleost species and the African lungfish Protopterus annectens. FEBS J. 273: 2839-2850.

97 For example, ADCs for copper were 73.2 ± 1.9 and 47.7 ± 3.7 for fish receiving 0% and 27% BPM, respectively; the corresponding values for phosphorus were 55.0 ± 0.7 and 63.9 ± 0.6, respectively.

98 Energy cost of maintenance and activity was calculated as the difference between heat loss and heat increment.

99 Heat increment was calculated as the difference in heat loss between fed fish and fasted fish.

respectively). Notable reductions in these parameters were observed only in the trout fed diets containing 27% BPM. The ADCs calculated for the sum of amino acids and lipids were statistically significantly lower than the respective controls only in salmon receiving the diet containing 27% BPM.

The ADCs for the sum of amino acids and individual amino acids were greater than the corresponding ADCs for nitrogen digestibility in trout, as Aas et al. (2006a) reported for salmon, indicating that the protein fraction is digested more effectively than non-protein fraction. These authors noted the potential for the cell wall to reduce the nitrogen digestibility of BPM compared with that of fish meal. However, BPM had no significant effect on digested lipid, energy, nitrogen or amino acids retention in trout at any test concentration, compared with the elevated retention of nutrients reported in salmon by Aas et al. (2006a).

6.1.3 Immunogenicity

Generally, dietary proteins affect the immune-system status of gut associated lymphoid tissue (GALT) when ingested. Accordingly, unpublished subchronic oral exposure studies in rats have demonstrated that BPM can cause moderate elevations of mesenteric lymph node (MLN) weight and macrophage/neutrophil levels of MLNs, which suggests stimulation of the innate immune system.[100] However, BPM levels less than 15% in the diet yielded minimal-to-no evidence of colonic or intestinal inflammation, no indication of severe colitis or tissue destruction, and no signs of secondary endotoxemia, pain, distress, or overt inflammation in these studies.

Christensen et al. (2003) showed that BPM-specific total Ig, IgA, IgG1, and IgG2A antibodies were elevated in blood samples and BPM-specific IgA antibodies were elevated in saliva samples from mice exposed to BPM in the diet.[101] They noted also that: (1) IgG1 antibody production is supported by T-helper cell type 2 (Th-2 cells) of humoral immunity, and (2) IgG2A antibody production is supported by Th-1 cells of cell-mediated immunity. The sustainment of IgG2A antibody levels observed after the cessation of exposure to BPM in this study suggests that factors supporting the Th-1 response in these mice may be cleared less efficiently than those supporting the Th-2 response. Th-1-type cytokines tend to produce pro-inflammatory responses that can lead to tissue damage if excessive. Th-2-type cytokines, on the other hand, are associated with anti-inflammatory responses. Thus, an optimum response to an immune challenge is generally a balanced Th-1 and Th-2 response.

Furthermore, IgG2A antibodies may have affinity for lipopolysaccharides (LPS). Christensen et al. (2003) noted that the main bacteria of BPM (*M. capsulatus*) contains LPS as an integral part of the cell membrane, which are likely candidates as adjuvants in BPM. However,

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[100] Glerup (1999). Scantox test report, prepared for Dansk BioProtein AIS, Lab No. 30864, 20 September, 73 pp; Svendsen & Damm Jorgensen (1992). Scantox test report, prepared for Dansk BioProtein A/S, Lab. No. 12960, July 31, 91 pp; Takawale (2004); Scantox test report, prepared for Norferm AIS, Study no. 52692, 20 October, 166 pp.; Thestrup (2004). Internal report, Norferm, 14 October, 12 pp.

[101] Christensen HR, Larsen LC, Frokiaer H (2003). The Oral Immunogenicity of BioProtein, a Bacterial Single-Cell Protein, is Affected by its Particulate nature, Brit. J. Nutr. 90: 169-178; WBC-specific total Ig, IgG_1, and IgG_{2A} antibodies were not measured in the saliva samples in this study.

Christensen et al. (2003) explained that LPS, which are abundant in the mucosal lumen, can enhance oral tolerance rather than potentiate the immunogenicity of an antigen. On the other hand, the sustained elevation of IgG2A antibody levels, accompanied by the decrease in IgG1 antibody in the blood of mice after the cessation of BPM exposure, as reported by Christensen et al. (2003), suggests the potential that chronic exposure to BPM in the diet may pose a risk for long-term inflammatory responses in mammalian species.

As noted above (Section 6.1.2. Salmonid Species), FeedKind® has been tested in Atlantic Salmon in one freshwater and two saltwater feeding studies published in a peer-reviewed journal.[102] Among these studies, Storebakken et al. (2004) showed that there were no histopathological changes in the anterior intestines, pyloric sacs or posterior intestines of Atlantic salmon fed up to 37% BPM in the diet starting at the fry stage and for 252 days thereafter in freshwater. As well, there were no histopathological changes observed in the cross section of the carcass (muscle, skin, kidney) or the liver, except for reduced hepatocellular vacuolization in the fish fed 37% BPM. Likewise, Berge et al (2005) found no histopathological changes in the intestines of Atlantic salmon fed up to 20% BPM in the diet starting from 1.39 kg initial body weight and for 150 days thereafter in seawater. Only one fish fed 10% BPM exhibited severely inflamed intestinal mucosa, leucocyte infiltration of the mucosa, and absence of absorptive vacuoles, but without the reduction in mucosal-fold height typical of the immunogenic response induced by soy protein in these fish. Furthermore, Aas et al. (2006) found increased copper concentrations in the viscera of salmon fed 36% BPM in the diet for 48 days in seawater, but no adverse effects on growth and survival, no effects on copper contents of any other tissues or on energy contents of viscera or any other tissue. These authors attributed the elevated copper levels in the viscera to the copper levels of the basal diet (5 ppm) plus the greater copper content BPM (87.9 ppm) compared to the fish meal it replaced (3.7 ppm). Overall, these studies showed that chronic exposure to diets containing up to 37% BPM does not induce an inflammatory response in the intestines of salmon, in contrast to soybean meal extracts used as a protein source in salmonid aquaculture.

Two scientific studies published in the peer-reviewed literature were performed to assess the potential for dietary BPM to produce immunogenic or immunotoxicological effects in Atlantic salmon. These studies are summarized below.

Romarhein *et al.* (2011) fed triplicate groups of juvenile Atlantic salmon (n=75/group; initial mean body weight = 133 g/fish) control fish-meal diet (*i.e.* 0% solvent-extracted soybean meal [SBM] and 0% BPM) or a diet in which the fish meal was incrementally replaced to contain 20% SBM, 30% BPM, or 20% SBM plus 30% BPM for 80 days.[103] Conventional solvent-extracted SBM is considered to be a suitable protein source for farmed salmonids, although dietary inclusion levels as low as 7.6% are known to cause SBM-enteritis in salmonid species, which is characterized by inflammation of the distal intestines. The mechanism for this reversible effect appears to involve

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[102] Storebakken T, *et al.* (2004) Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo salar*, in freshwater. *Aquaculture*. 241: 413-425; Berge GM, *et al.* (2005) Bacterial protein grown on natural gas as protein source in diets for Atlantic salmon, *Salmo salar*, in saltwater. *Aquaculture*. 244: 253-240; Aas TS, et al. (2006a) Improved growth and nutrient utilization in Atlantic salmon (Salmo salar) fed diets containing a bacterial protein meal. Aquaculture. 259: 365-376.

[103] Romarheim OH, *et al.* (2011) Bacteria grown on natural gas prevent soybean meal-induced enteritis in Atlantic salmon. *J Nutr*. 141: 124-130.

impaired immune tolerance to SBM caused by alcohol-soluble components of SBM, such as saponins.

As expected, the fish fed 20% SBM for 80 days in this study developed enteritis, lacked carbonic anhydrase 12 in the epithelial cells of the brush border of the distal intestines,[104] and had greater numbers of epithelial cells reacting to proliferating nuclear antigen compared with the fish fed the other diets. The fish fed control, 30% BPM, or 20% SBM plus 30% BPM showed no signs of inflammation in the distal intestines on histopathological examination of the tissues.

Furthermore, the fish fed 20% SBM in the diet exhibited statistically-significantly reduced final body weight, thermal growth coefficient (TCG), and FCR, compared to fish fed the control diet. On the other hand, 30% BPM in diet resulted in a statistically significant increase in FCR but no statistically significant differences in final body weight or TCG.[105]

Digestibility of crude protein and lipids was statistically-significantly reduced in the fish receiving 30% BPM in the diet, compared with controls,[106] but total gut, liver, stomach, and mid- and distal-intestine weights, were elevated relative to body weight in these animals.[107]

The authors concluded that BPM counteracts or neutralizes SBM-induced enteritis in Atlantic salmon.

In a follow-on study (Romarhein *et al*., 2012), duplicate groups of juvenile Atlantic salmon (n=50/group; initial mean body weight = 273 g/fish) a control fish-meal diet (*i.e.* 0% SBM and 0% BPM) or a diet in which the fish meal was incrementally replaced to contain 20% SBM plus 0%, 2.5%, 5%, 10%, 15%, 20% or 30% BPM for 47 days.[108] Only one fish died after the experiment was started.

Fish fed 20% SBM in the diet developed SBM-enteritis, as expected, but this effect decreased with increasing BPM levels in the diets containing 20% SBM. Likewise, the number of clusters of $CD8\alpha^+$ intraepithelial lymphocytes in fish fed 20% SBM decreased with increasing BPM inclusion levels.[109] Morphometric evaluation revealed that intestinal stretches stained for proliferating-cell nuclear-antigen in the fish fed 20% SBM plus ≥15% BPM were indistinguishable

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[104] Carbonic anhydrases (CAs) are zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide, participate in a variety of biological processes, and are highly expressed in normal tissues.

[105] For example, final body weights were 362, 319, 344, and 328 g/fish for fish fed the control, 20% SBM, 30% BPM, and 20% SBM plus 30% BPM diets, respectively.

[106] Mean digestibility of crude protein was 86.6%, 86.1%, 83.3%, and 84.6% and mean digestibility of crude lipid was 96.1%, 94.7%, 92.2%, and 95.7% for fish fed the control, 20% SBM, 30% BPM, and 20% SBM plus 30% BPM diets, respectively.

[107] For example, mean relative liver weights were 16.1, 15.3, 17.2, and 19.3 g/kg body weight for fish fed the control, 20% SBM, 30% BPM, and 20% SBM plus 30% BPM diets, respectively.

[108] Romarheim OH, *et al.* (2012). Prevention of soya-induced enteritis in Atlantic salmon (*Salmo salar*) by bacteria grown on natural gas is dose dependent and related to epithelial MHC II reactivity and $CD8\alpha^+$ intraepithelial lymphocytes. *Br J Nutr*. 109 (6): 1062-1070.

[109] Mobilization of CD8α+ T cells indicates that SBM-induced enteritis is a T-cell-mediated inflammatory response to SBM.

from those of fish fed the control diet, as was the number of clusters of $CD8\alpha^+$ intraepithelial lymphocytes at the base of the intestinal epithelium in fish fed 20% SBM plus ≥20% BPM. Staining for major histocompatibility complex class II (MHC II) revealed numerous reactive leucocytes in the brush border and other areas of the intestinal epithelium in salmonids fed 20% SBM plus 0%, 1.2%, or 5% BPM, but this effect decreased in a concentration-dependent manner in salmonids were fed 20% SBM plus 10%, 20% or 30% BPM. Single and isolated lymphocyte aggregates consisting primarily of leucocytes were found in otherwise normal intestines in 2 of 12 and 1 and 12 fish fed 20% SBM plus 20% BPM and 20% SBM plus 30% BPM, respectively.

There were no significant differences in feed consumption or growth across all groups, although 20% and 30% BPM in the diet were associated with slightly reduced protein digestibility[110] and increased relative weight of the distal intestines.[111] The authors suggested that the continued increase in the relative weights of the distal intestines at dietary BPM levels greater than levels that prevented SBM-induced enteritis indicates that BPM stimulates intestinal growth in the affected fish. This could be because, for example, BPM contains relatively high purine and pyrimidine levels that can serve as substrates supporting the growth of intestinal epithelial cells.

As in the previous experiment, there was no effect on the mean relative weights of the total gut, liver, stomach, pyloric region or mid-intestines. The authors suggested that the mechanism by which BPM counteracts pro-inflammatory responses in salmonids exposed to 20% SBM in the diet is related to immune-system mechanisms that are also responsible for ensuring tolerance to feed antigens and to commensal intestinal microbiota.

Overall, the results of studies of salmonid species chronically exposed to up to 37% BPM in the diet are uniformly negative for any signs of an inflammatory response that can be attributed to BPM exposure. These studies demonstrate that chronic exposures to BPM, even at very high levels in the diet, do not produce the exposure-related chronic inflammatory responses suggested based on the changes in antibody titers reported in mice orally exposed to BPM.

Appendix 14 includes the statement of Drs. Judith T. Zelikoff and Daniel Wierda, experts in the field of fish and mammalian immunotoxicology, which addresses the elevation in serum IgG2a levels in mice and, this, the potential for long-term dietary exposure to FeedKind® to pose a risk of chronic inflammation in salmonids. These experts stated that it cannot be concluded that increases in IgG2 levels in mammals or a possible equivalent in fish will, or even could, lead to an inflammatory response. They noted that a postulated humoral immune-system response in fish fed diets containing FeedKind® (equivalent to BPM) is not likely to be analogous to the production of IgG2A reported in mice by Christensen et al. (2003) and, in any case, does not appear to be associated with consequent adverse inflammatory processes in fish. They concluded that long-term dietary exposure to FeedKind® does not pose a risk of chronic inflammation in salmonids and that, therefore, FeedKind® is safe for use at concentrations up to 18% in the diet of salmonids, based on

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110 The authors suggested that the reduced protein digestibility of BPM is attributable to cell-wall components of BPM, as it is for yeast products fed to salmonids, as well as the extensive intracytoplasmic membranes of M. capsulatus grown on natural gas.

111 Mean crude-protein digestibility was 83.7%, 84.9%, 84.1%, 84.5%, 83.8%, 84.8%, 83.6%, and 82.2% in fish fed 20% SBM plus 0%, 2.5%, 5%, 10%, 15%, 20% or 30% BPM, respectively, for 47 days; corresponding average distal-intestine-to-body-weight ratios were 5.8, 5.6, 5.2, 5.6, 6.3, 6.1, 6.8, and 7.0, respectively.

their review of published rodent and salmonid studies and supporting unpublished studies, as well as the acknowledgement of differences in the mechanisms of immune responses in mammals compared with bony fish.

6.1.4 Liver weight and prothrombin time

In two Scientific Opinions published in 2017, the European Food Safety Authority (EFSA) Panel on Additives and Products or Substances in Animal Feed (FEEDAP) expressed uncertainties about the safety of genetically-modified (GM) *Escherichia coli* (*E. coli*) and other gram-negative bacterial biomasses intended to be fed to food producing mammalian species.[112] Specifically, the EFSA Panel noted that the mechanisms for the increased liver weights reported in pigs and reduced prothrombin time reported in multiple species fed biomasses produced by the GM *E. coli* (gram negative) strains are not known. However, the Panel acknowledged that these effects were small and clearly not attributable to the systemic absorption of endotoxins or lipopolysaccharides (LPS) from these biomasses in the digestive tracts of the animals tested, and dietary variation is a plausible explanation for the effects reported.

The Panel noted that other products derived from other gram-negative microorganisms may pose similar issues, without citing evidence to implicate gram-negative organisms other than *E. coli* as having any potential to cause adverse effects, and without providing any specific rationale for this assertion. On the contrary, the Opinions of the EFSA Panel provided few or no assertions that the *E. coli* biomasses would have adverse effects on the animals or on humans ingesting products derived from food-producing animals fed these biomasses. The Panel's conclusions in both Opinions stated that "the recipient strain E. coli K-12S B-7 is considered to be safe."

Like *E. coli*, the *M. capsulatus* that serves as an integral microorganism of the consortium used to produce FeedKind® is a gram-negative bacterium. However, there is no evidence in any of the numerous, substantial studies that have been performed with BPM, equivalent to FeedKind®, in pigs, rats, and other mammalian species, as well as in salmonids, suggesting that short-term or long-term exposures to FeedKind® is inherently dangerous or unsuited for use in salmonids at the proposed feeding levels for these endpoints (i.e. increased liver weight or decreased prothrombin time). There are biological, physiological and taxonomic differences between *M. capsulatus* and *E. coli*. There is no evidence that *M. capsulatus* produces harmful endotoxins, lipopolysaccharides (LPS), or any other substance identical or similar to such substances produced by some strains of *E. coli* and other gram-negative microorganisms that are known to be pathogens. There is no evidence in the literature implicating *M. capsulatus* as having any pathogenic, toxic or other negative characteristics, as discussed in Section 6.1.1.

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[112] EFSA FEEDAP Panel (2017). Scientific Opinion on the safety and nutritional value of a dried killed bacterial biomass from *Escherichia coli* (FERM BP-10941) (PL73 (LM)) as a feed material for pigs, ruminants and salmonids. EFSA Journal. 15:4935. Available at: https://doi.org/10.2903/j.efsa.2017.4935; EFSA FEEDAP Panel (2017). Scientific Opinion on the safety and nutritional value of a dried killed bacterial biomass from *Escherichia coli* (FERM BP-10942) (PT73 (TM)) as a feed material for pigs, ruminants and salmonids. EFSA Journal.15:4936. Available at: https://doi.org/10.2903/j.efsa.2017.4936.

Furthermore, gram-negative microorganisms are used as fish feed or to produce fish feed substances. For example, a species of *M. extorquens* is GRAS for use in fish feed (product name: KnipBio; AGRN26).

6.1.5 Human toxicity

None of the substances in FeedKind® fed to animals is expected to be transferred, intact, to people consuming the edible products of any of the food-producing animals. The composition of FeedKind® is like that of other common animal feeds, including amino acids, phospholipids, and lipopolysaccharides. Therefore, FeedKind® consumed by the target animals will be digested and converted to biomass and as such there is no expected exposure to FeedKind® for humans via consumption of target animals. There is no evidence indicating that the nucleic acids in FeedKind® would be incorporated into human food products to be transferred to consumers. There is no evidence that any hazardous substances are formed during the production of FeedKind®. Further, the effects observed in studies in which animals were fed diets containing relatively high concentrations of FeedKind® (generally reduced growth rates and final body weight) are not expected to affect the health of people consuming such products. Thus, the human health risks associated with the consumption of products from animals fed FeedKind® are negligible at the dietary concentrations tested in the studies summarized above.

6.1.6 Conclusion

Table 16 presents the NOAELs and LOAELs from the key safety studies summarized above to support specific FeedKind® inclusion levels in diets for salmonid species, together with brief statements of the adverse effects observed at each LOAEL.[113]

[113] The critical effect is defined as the first adverse effect, or its known precursor, that occurs in a test species as the dose or exposure concertation increases.

Table 16. Safety Study Summaries for FeedKind® (BPM) Inclusion Rates

Species	NOAEL (% of diet)	LOAEL (% of diet)	Exposure Duration (days)	Critical Effect(s)	Ref
Atlantic salmon	19.3	37	364	Reduced body weight, specific growth rate, and survival rate	Storebakken *et al.* (2004)
Atlantic salmon	20	N/D*	150	None	Berge *et al.* (2005)
Atlantic salmon	36	N/D*	48	None	Aas *et al.* (2006a)
Rainbow trout	18	27	71	Reduced apparent digestibility coefficients for N, lipid, energy, amino acids; elevated energy used for activity and maintenance	Aas *et al.* (2006b)

*N/D = not determined; there were no adverse health effects observed at the highest dietary FeedKind® inclusion rate tested.

The most conservative species-specific NOAELs determined from the results of the key feeding studies include the following:

- Atlantic salmon: 19.3%
- Rainbow trout: 18%

The results of the studies indicate that the NOAEL for Atlantic salmon during the saltwater phase of its lifecycle is 36%, which is nearly twice as high as the NOAEL observed during the early or juvenile freshwater phase. The lower value reported for Atlantic salmon during the freshwater phase can be attributed to testing with a feed pellet size too large for the size of the fish and other experimental conditions, rather than to effects attributable specifically to the FeedKind® in the feed. Nevertheless, Calysta utilizes the most conservative NOAEL derived from well-conducted, well-reported studies, including a chronic- and two subchronic-exposure studies on Atlantic salmon and a subchronic-exposure study on rainbow trout to conclude that the studies summarized above support specific FeedKind® inclusion levels up to 18% in diets for salmonid species.

Therefore, we believe the above summarized data fully supports the safe use of FeedKind® at inclusion rates not to exceed 18% in salmonid species.

6.1.7 Summary of safety argument; assertion of GRAS status

Calysta concludes that the generally available data and information that establish safety, as discussed above, provide a basis that the notified substance is generally recognized among qualified experts to be safe under the conditions of its intended use for the target animal species and for humans consuming human food derived from food producing animals.

The notified substance is a fermentation of naturally occurring microorganisms that have not been reported to be a safety concern in the company's literature searches. Manufacture of FeedKind® will use a consistent growth medium with standard fermentation procedures. Raw materials of suitable purity will be used in manufacture and manufacture will be occur under Good Manufacturing Practice. There are not expected to be any impurities in FeedKind® relevant to the health or safety of the target species to which FeedKind® will be fed. Finally, the safety studies conducted by Calysta and others indicate that the product is safe for use at the level contemplated.

Use of FeedKind® will not result in any adverse health effects in humans consuming animals that have been fed FeedKind®, because humans will not be exposed to any unique components or compounds. FeedKind® will be fully metabolized by the target species and, when incorporated in the flesh of the target species, the amino acids and other components of FeedKind® will be indistinguishable from the same components derived from other sources. There are not expected to be any impurities that would impact the target species or which would persist in the flesh of the target species and pose a risk to human health when consumed. Based on the above, Calysta concludes that the notified substance is Generally Recognized as Safe for use in aquaculture feed for salmonid species when used as an additive of up to 18% by weight in the animal feed.

6.2. Basis for GRAS conclusion for intended use of FeedKind®

As described above, the safety of FeedKind® for use in salmonid feed at the levels indicated within this submission is demonstrated by published and supported by unpublished toxicity studies and in supportive feeding studies.

6.3. Safety of constituents

FeedKind® is the only constituent for which a GRAS Notice is being submitted. Safety of FeedKind® has been addressed above.

Part 7 – List of supporting data and information

Calysta has disclosed all safety data of which it is aware and have found none that is inconsistent with the GRAS determination.

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APPENDICES

Appendix 1. Genome Sequencing Report for DB3, DB4, DB5

Appendix 2. n-Hexane, Cyclohexane, Benzene and Toluene Safety Assessment

Appendix 3. Mercury Concentration Assessment

Appendix 4. Nitric Acid Metals Content

Appendix 5. Ash Content Data

Appendix 6. Method Summary S1172 The Determination of Heavy Metals by ICP-MS

Appendix 7. Nickel by ICP-MS Validation Summary

Appendix 8. (b) (4) **Analytical Methods – Summary**

Appendix 9. Tyrosine PT Data 2019

Appendix 10. Stability Testing Report

Appendix 11. Method Summary – S2106 – Yeasts and Moulds

Appendix 12. Historic Stability Testing

Appendix 13. 1995 Scientific Committee for Animal Nutrition Report

Appendix 14. Statement of Drs. Judith T. Zelikoff and Daniel Wierda

CALYSTA REPORT

2019-10-28

Genome Assembly of DB3, DB4, DB5

By (b)(6)

Table of Contents

Summary

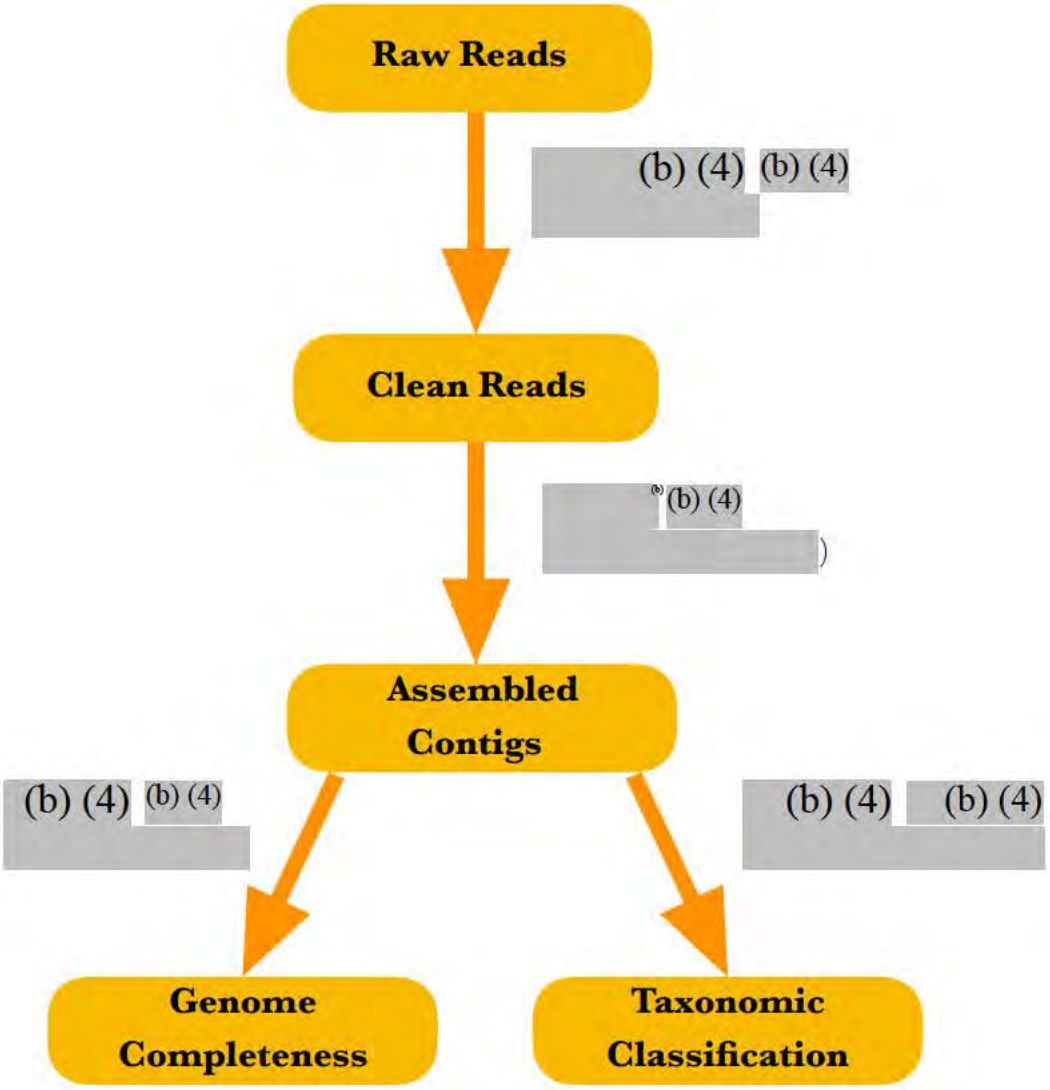
The datasets consisted on three genomes (**DB3**, **DB4**, and **DB5**). Each of these genomes was sequenced by (b) (4) using the (b) (4), with 2x150bp reads.

This reports shows the results of the *de novo* assembly and phylogenomic classification of the three genomes. In addition, each genome was compared to a previously selected reference genome (**DB3**, *Cupriavidus gillardii*. **DB4**, *Brevibacillus brevis*. **DB5**, *Brevibacillus agri*).

Using *de novo* assembly, we were able to assemble the three genomes with good results. Based on the presence of single copy gene markers all of the genomes were sequenced to completion. Phylogenomics analysis allowed the taxonomic classification of each genome to the best possible taxonomic category. **DB3** was classified up to the genus level (*Cupriavidus*). **DB4** to the species level, where the most similar species was *Anaeurinibacillus* sp002375825, a genome assembled from a metagenome dataset but with no reported isolate. **DB5** was classified up to the species level, as *Brevibacillus agris*.

Average nucleotide identity (ANI) analysis of the three genomes with the selected references, showed that **DB3** had a 91.95% mean ANI value with *Cupriavidus gillardii*, supporting the result that **DB3** is a new species within the *Cupriavidus* genus. **DB4** had a 74.61% mean ANI value with *Brevibacillus brevis*, which strongly suggest that both genomes come from taxonomically unrelated microorganisms. **DB5** had a 99.56% mean ANI value, supporting the classification of this genome as a strain of *Brevibacillus agri.*

Analysis Workflow



Genome Assembly Results

Table 1 shows the assembly results for the three genomes. Overall, the assembly results are good. Because all of the genomes were sequenced using short reads ((b) (4) sequencing), is not possible to assembled them into a single chromosome.

Table 1. Genome assembly statistics

	DB3	DB4	DB5
Total N° contigs	673	470	520
Total N° contigs, over 500 bp.	71	178	160
Largest contig	375,320	154,135	446,056
Total length (all contigs)	5,930,539	4,589,507	5,492,175
Total length (contigs over 500bp.)	5,777,668	4,521,391	5,407,915
N50	163,990	52,744	84,594
L50	12	27	15
%GC	68	46.61	53.7

Based on the (b) (4) (b) (4) results (which checks for the presence of single copy genes in the assembled genome), all of the genomes were sequenced to completion (Table 2), with low levels of contamination, which suggest that in all of the cases there was no contamination from another organism in the sequence information.

Table 2. (b) (4) results

	DB3	DB4	DB5
Genome completeness (%)	99.89	99.20	99.73
Contamination (%)	4.96	1.64	1.6

Taxonomic Analysis

Taxonomic classification of each genome was done using (b) (4) (b) (4) which uses 120 bacterial markers to perform a phylogenomic analysis and compare the genome of interest against a collection of 145,904 genomes from the Genome Taxonomy Database (Parks, Chuvochina et al. 2018). The results (Table 3)

	DB3	DB4	DB5
Phylum	Proteobacteria	Firmicutes	Firmicutes
Class	Gammaproteobacteria	Bacilli	Bacilli
Order	Burkholderiales	Anauerinibacillales	Brevibacillales
Family	*Burkholderiaceae*	*Anaeurinibacillaceae*	*Brevibacillaceae*
Genus	*Cupriavidus*	*Aneurinibacillus*	*Brevibacillus*
Species		*Aneurinibacillus* sp002375825	*Brevibacillus agri*

DB3

In the case of **DB3**, the best classification that can be achieved using this method was up to Genus level, in this case to *Cupriavidus*. This suggest that this genome may represent a novel species within the *Cupriavidus* genus, as no similar species was found using a phylogenomic approach.

An additional comparison against a reference genome for *Cupriavidus gilardii* was performed, to confirm that **DB3** is indeed a different species. Figure 1 shows the mapping of all **DB3** against the genome of *C. gilardii* CR3 .

The alignment shows that although there are reads that map to the genome, the coverage is uneven (in particular in the second chromosome). In addition the coverage is a multiple levels of sequence identity (from 75% going up), suggesting that the genomes are similar (probably from the same genus), but are not closely related. For example, if we want to classify **DB3** as a strain of *C. gilardii*, we should expect a higher and even coverage of all the genome (both chromosomes), and also at higher identity levels (ideally close to a 100%). This is not observed on Figure 1, supporting the phylogenomic results that suggest that **DB3** is a novel species within the *Cupriavidus* genus.

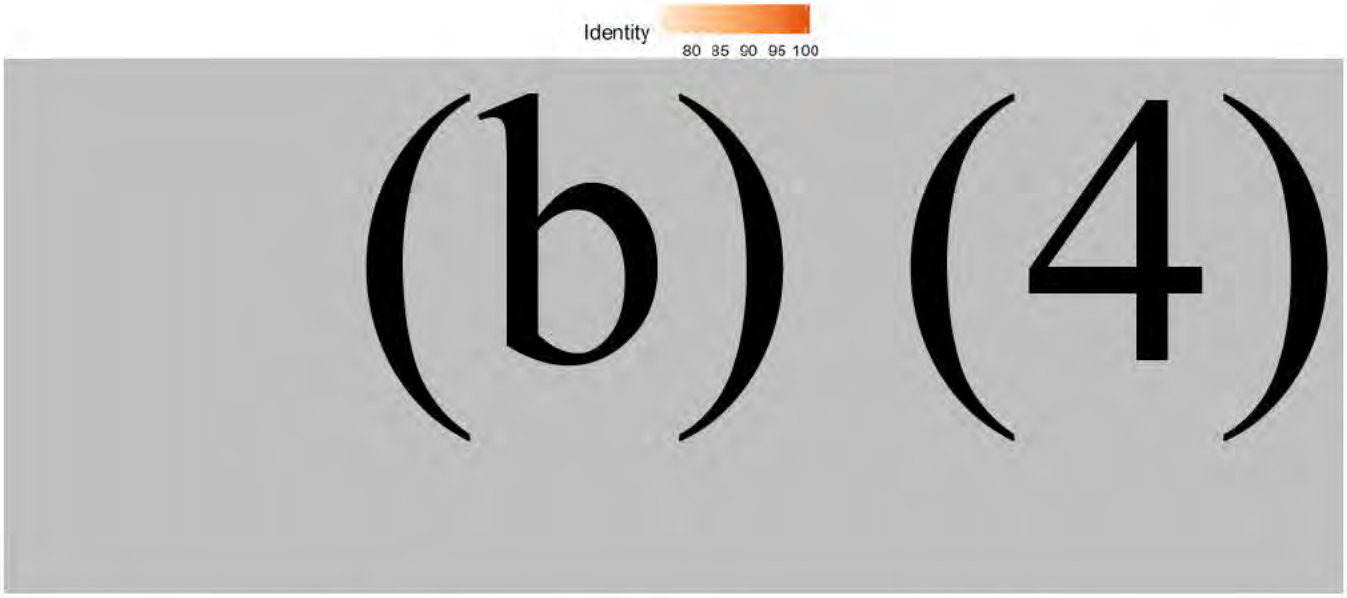


Figure 1. Mapping of the **DB3** reads against the two chromosomes of *C. gilardii*.

Another analysis that can be performed to confirm the observation that **DB3** is a novel species, is to directly compare their genome sequences. Using a metric called average nucleotide identity (ANI) (Goris, Konstantinidis et al. 2007), we can compared both genomes and determine the average nucleotide identity between them. In general, values above 95% can be considered to be genomes that belong to the same species. In this case (Figure 2), the ANI value between **DB3** and *C. gilardii* is only 91.95%, also supporting the classification of **DB3** as a novel species within the *Cupriavidus* genus.

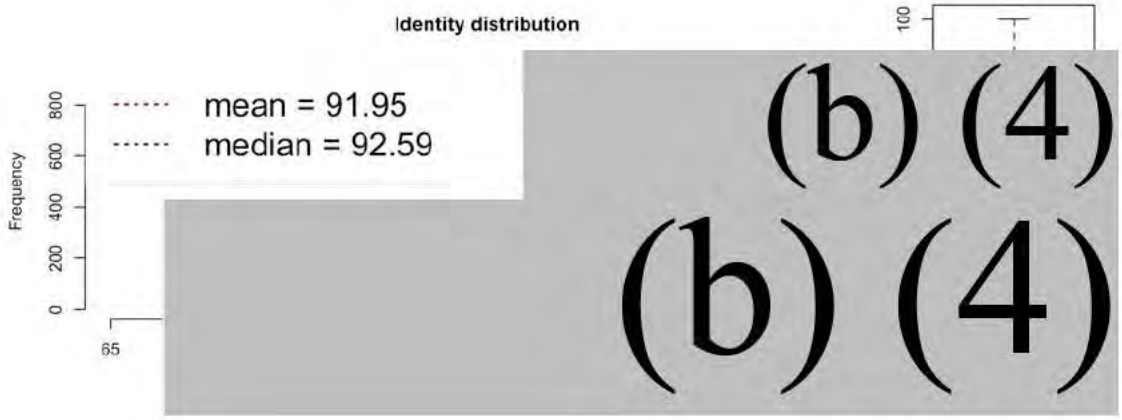


Figure 2. Distribution showing the frequency of hits at different identity thresholds for the genomes of **DB3** and *C. gilardii*. The results shows that the mean average nucleotide identity between the two genomes is 91.95%.

DB4

The phylogenomic analysis allowed classification of this genome up to the species level. The closest species found was a genome called *Anaeurinibacillus* UBA3580. This correspond to a metagenome-assembled genome, a sequence obtained by assembling and curating metagenomic datasets, and not from an actual isolate. This genome was generated in a recent study (Parks, Rinke et al. 2017), by assembling more than 8,000 metagenomic samples. Because of the large-scale nature of that study, it is difficult to track the origin of the sample were *Anaeurinibacillus* UBA3580 was obtained. More information about that genome can be found on its NCBI page (https://www.ncbi.nlm.nih.gov/assembly/GCA_002375825.1).

ANI analysis (Figure 3), supports the phylogenomic findings. The ANI value between the two genomes is 99.54%, which strongly suggest that they are the same species. Based on this evidence, **DB4** represents (based on the available information) the first isolate for this species. In contrast, when the comparison was performed between **DB4** and *Brevibacillus brevis*, the ANI value between the two genomes was 74.61%, which is outside the detection limit of this approach, showing that these two genomes are unrelated at the species and probably at the genus level as well.

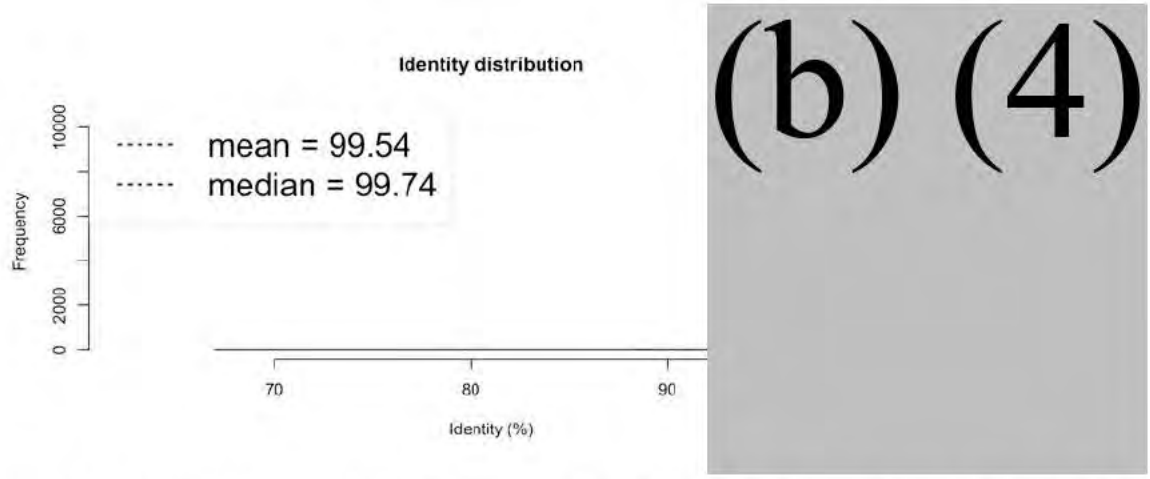


Figure 3. Distribution showing the frequency of hits at different identity thresholds for the genomes of **DB4** and *Anaeurinibacillus* UBA3580. The results shows that the mean average nucleotide identity between the two genomes is 99.54%

DB5

The phylogenomic analysis allowed classification of this genome up to the species level. The closest species match was to *Brevibacillus agri*. This is the same species used as a reference.

Comparing the *B. agri* genome with **DB5** (Figure 4), shows that the ANI value between both genomes is 99.56%, supporting the phylogenomic results and allowing the classification of **DB5** as a strain of *B. agri*.

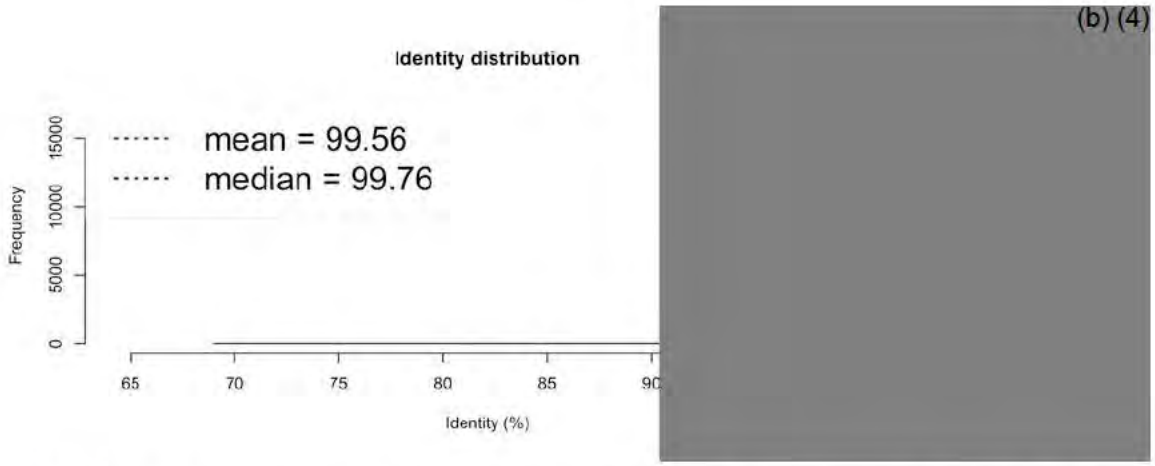


Figure 3. Distribution showing the frequency of hits at different identity thresholds for the genomes of **DB5** and *B. agri*. The results shows that the mean average nucleotide identity between the two genomes is 99.56%

Software packages

- (b) (4)

References

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Goris, J., K. T. Konstantinidis, J. A. Klappenbach, T. Coenye, P. Vandamme and J. M. Tiedje (2007). "DNA DNA hybridization values and their relationship to whole-genome sequence similarities." International journal of systematic and evolutionary microbiology **57**(1): 81-91.

(b) (4)

Parks, D. H., M. Chuvochina, D. W. Waite, C. Rinke, A. Skarshewski, P.-A. Chaumeil and P. Hugenholtz (2018). "A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life." Nature biotechnology **36**: 996-1004.

(b) (4)
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Parks, D. H., C. Rinke, M. Chuvochina, P.-A. Chaumeil, B. J. Woodcroft, P. N. Evans, P. Hugenholtz and G. W. Tyson (2017). "Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life." Nature Microbiology **2**(11): 1533-1542.

Appendix 2

n-Hexane, cyclohexane, benzene and toluene Safety Assessment

Chao and Attari (1995) reported the results of a 3-year program performed to survey the detailed compositions of pipeline gas from major producing areas in the US, imported natural gas from Canada, and natural gas used to generate electricity at 4 power plants in the U.S. The natural gas stream samples were collected from 19 separate sampling points (including 4 power plants) in 10 states across the continental U.S. from October 1990 to 1993.[1] The origins of the natural gas sampled ranged from the on-shore and off-shore Gulf coast to Northern California and Canada. The samples were analyzed to measure the concentrations of a comprehensive list of major, minor, and trace constituents utilizing a complete field sampling and analysis system that had been developed and validated, including proportional sampling, cryogenic sampling, sorbent sampling, and on-line measurement techniques. The method detection limits (MDLs) included 0.1 ppmv for toluene and 0.2 ppmv for benzene, hexanes, and cyclohexane.[2] Table 1 presents the summary statistics for these natural gas constituents.

Table 1. Summary Data for Selected Natural Gas Constituents Reported by Chao and Attari (1995)[3]

Constituent(s)	# of Samples	Range (ppmv)	Median (ppmv)	Sample ID
Hexanes	19	<0.2 to 1156	170	IGT-041
Cyclohexane	17[4]	<0.2 to 146	24	IGT-082
Benzene	17	<0.2 to 471	7	IGT-022
Toluene	17	<0.1 to 100	6	IGT-022

The samples with the highest concentrations of these analytes were IGT-041 for hexanes, IGT-082 for cyclohexane, and IGT-022 for benzene and toluene.

Black and Veatch reported the compositions of 3 examples of pipeline quality natural gas from US-based interstate facilities in the year 2000 and later, which illustrate the range of natural

1 Chao and Attari (1995), Figure 1, page 12.

2 Chao and Attari (1995), Table 1, page 8.

3 Chao and Attari (1995), Table 7, page 50;

4 Cyclohexane was not measured in 2 of the 19 samples, identified as IGT-011 and IGT-012, which were the 2 samples analyzed the earliest in the survey. BTEX analytes were measured in these 2 samples but, like cyclohexane, BTEX is not included in the summary statistics. The reason for this is that a relatively low resolution GC column was used for these earliest analyses, so that cyclohexane, C8 hydrocarbons, and C9 hydrocarbons were not adequately separated from benzene and toluene, respectively. The issue was remedied to measure these analytes in the subsequent 17 samples.

gas compositions that meet minimum pipeline specifications for consumer use.[5] The concentrations of constituents presented for these examples are consistent with those reported by Chao and Attari (1995). Specifically, the concentration ranges reported were:

- Benzene: 44 to 470 ppmv
- Toluene: 18 to 100 ppmv

In the screening-level safety assessment presented below, we assumed that the concentration of each natural gas constituent is the maximum value reported in Chao and Attari (1995), which equaled or exceeded the corresponding maximum concentrations reported by Black & Veatch. The concentrations considered for the screening assessment are presented in **Table 2**.

Table 2. Maximum Reported Concentrations of Selected Natural Gas Constituents

Constituent(s)	Concentration (ppmv)	Concentration (ppmw)[6]	Sample ID
Hexanes	1156	5009	IGT-041
Cyclohexane	146	695	IGT-082
Benzene	471	1924	IGT-022
Toluene	100	477	IGT-022

Like methane, all of the constituents listed in Table 6 are susceptible to substantial metabolic degradation by *M. capsulatus* (Bath) and will be reduced substantially during the manufacturing of FeedKind®. For example, Colby *et al.* (1977) demonstrated the very broad substrate specificity that the methane mono-oxygenase of *M. capsulatus* possesses, which catalyzes a variety of different oxygen-incorporation reactions.[7] Colby *et al.* (1977) showed that this mono-oxygenase effectively catalyzed the aerobic oxidation of C1, C2, C3, C4, C5, C6, C7 and C8 n-alkanes with a specific activity of 85, 63, 68, 68, 69, 39, 27, and 9 milli-units/mg protein, respectively, to produce the corresponding alcohols. In addition, they showed that this enzyme catalyzed the metabolism of cyclohexane, benzene, and toluene under the same conditions with a specific activity of 62, 62, and 52 milli-units/mg protein, respectively, to yield cyclohexanol, phenol, and benzyl alcohol, respectively. Thus, the safety assessment calculations presented below

[5] Black & Veatch (2021). Natural Gas technical Paper. Prepared for Calysta, 7 pp.

[6] Conversion from ppmv to ppmw was accomplished by multiplying the concentration of each constituent (ppmv) in a natural gas sample by its molecular weight and adding the products of the multiplications together, then dividing the product of each constituent by the sum of the products and multiplying the result by 10^6.

[7] Colby J, Stirling DI, Dalton H (1977). The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath): Its ability to oxygenate n-alkanes, n-alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds. Biochem. J. 165: 395-402; For review see Jiang H, Chen Y, Murrell JC, Jiang P, Zhang C, Xing X-H, Smith TJ (2010). Methanotrophs: Multifunctional bacteria with promising applications in environmental bioengineering. Biochem. Engineer. J. 49:277-288.

considers the ability of *M. capsulatus* to metabolically detoxify n-hexanes, other n-alkanes, cyclohexane, and other aromatic constituents.

In addition, (b) (4) , where no less than 99.5% of the volatile organic carbon (VOC), including any benzene and toluene that may be present in the off-gas, is decomposed to yield carbon dioxide (CO_2). Thus, essentially all of the n-alkanes, including n-hexane, in the off-gas will be destroyed in the combustion chamber. Unlike the cyclic and aromatic VOCs including cyclohexane, benzene and toluene, methane and n-alkanes are not subject to potential induced-dipole to induced-dipole interactions with the aromatic amino acids of proteins. However, the loss of n-alkanes through off-gassing was not accounted for in the calculations summarized below, which contributes to the conservatism of calculations.

In sum, we assumed, conservatively, that:

- 100% of n-hexane, cyclohexane, benzene and toluene that enter the fermenter with natural gas during fermentation are present in the FeedKind® biomass after (b)(6)
- Cyclohexane, benzene and toluene may accumulate in the biomass because of potential induced-dipole to induced-dipole interactions with the aromatic amino acids of the proteins of the biomass
- 100% of the "hexanes" that enter the fermenter is present as n-hexane and can accumulate in the biomass, although n-hexane is:
    - Not susceptible to induced-dipole to induced-dipole interactions.
    - A well-known neurotoxicant at sufficiently high inhalation concentrations but has not been demonstrated to be neurotoxic to humans by ingestion or dermal exposure.

Furthermore, there are at least 2 steps of the FeedKind® manufacturing process that substantially reduce the potential levels of any residual hexane, cyclohexane, and benzene that may remain in the finished product, (b) (4)

(b) (4)

These values were calculated as follows:

- (b) (4)

(b) (4)

The enthalpies of evaporation and the boiling points of hexane, cyclohexane, and benzene are lower than the corresponding values for water, and the enthalpy of evaporation for toluene is lower than that of water, as shown in **Table 3**.[11]

Table 3. Boiling Points and Enthalpy of Vaporization of Selected Natural Gas Constituents

Constituent	Boiling Point (°C)	Molar Enthalpy of Vaporization (kJ/mol)
n-Hexane	68	31.5
Cyclohexane	80.7	33.5
Benzene	80.1	30.7
Toluene	110.6	38.1
Water	**100**	**40.7**

(b) (4)

(b) (4)

[11] Kotz JC, Treichel P (1999). Chemistry and Chemical Reactivity. 4th Edition, Saunders College Division.

Table 4 presents the maximum concentrations of selected natural constituents in finished FeedKind® and in salmonid food assumed in the screening level safety assessment calculations below.

Table 4. Maximum Concentrations of Natural Gas Constituents in FeedKind® and Salmonid Food

Constituent(s)	Natural Gas Sample ID	Maximum Concentration in Natural Gas (ppmw)	Percent Retained in FeedKind® (%)	Maximum Concentration in FeedKind® (ppm)[12]	Maximum Concentration in Finished Salmonid Food (ppm)[13]
Hexanes	IGT-041	5009	1.05%	(b) (4)	
Cyclohexane	IGT-082	695	1.05%		
Benzene	IGT-022	1924	1.05%		
Toluene	IGT-022	477	100%		

Experiments with salmon liver microsomes have demonstrated that salmonids have the capacity to metabolize cyclohexane, benzene and chemically related compounds and, therefore eliminate these compounds effectively and rapidly. For example, Kennish *et al.* (1988) demonstrated that hepatic enzymes of adult Chinook salmon metabolized toluene to yield benzyl alcohol with very similar kinetics as Kennish *et al.* (1985) described earlier for the metabolism of cyclohexane by Coho salmon liver enzymes to yield cyclohexanol under the same optimal conditions.[14] Kennish *et al.* (1988) noted that optimal conditions of temperature (15°C to 25°C), pH and ionic strength for the catalysis of cyclohexane and toluene were identical across salmon species tested in their studies. Kennish *et al.* (1985) noted that the optimal temperature (i.e. 20°C) yielding the maximum rate in salmon is substantially lower than the optimal temperature reported

(b) (4)

[14] Kennish JM, Gillis D, Hotaling K (1988). Metabolic conversion of toluene and ethylbenzene by Pacific salmon microsomal preparations. Mar. Environ. Res. 24: 69-71; Kennish JM, Montoya C, Whitsett J, French JS. (1985). Metabolic conversion of cyclohexane by Pacific salmon microsomal preparations. Mar. Environ. Res. 17: 129-132.

for mammalian systems, which is attributable to genetic, developmental and environmental factors.[15]

Roubal *et al.* (1977) reported that benzene was rapidly metabolized and eliminated from the bodies of young Coho salmon following intra-peritoneal (*i.p.*) injection of uniformly labelled ^{14}C-benzene (198 µCi/mg).[16] We estimate that the total dose of benzene administered to each fish in this study was approximately 12 mg/kg bw.[17] Injections *i.p.* bypass first-pass metabolism in the gut but not in the liver. Roubal *et al.* (1977) found only 0.066%, 0%, 0.02%, 0.01%, and 6.22% of the radioactivity administered to the fish in the flesh, brain, liver, gall bladder, and carcass, respectively, 6 hours post exposure (i.e. total ~6.3% of the administered dose remaining in the animals). Only 0.006%, 0%, 0%, and 0.22% of the radioactivity remained in the flesh, brain, liver, gall bladder, and carcass, respectively, 24-hours post-exposure. The results clearly demonstrated that benzene was readily metabolized in the liver and benzene and its metabolites were rapidly eliminated from the bodies of the fish after exposure.

Furthermore, sound U.S. and global aquacultural practices require fasting and feed withdrawal periods prior to slaughter. Benefits include complete gut evacuation, a clean digestive tract, good water quality by minimizing ammonia and fecal excretion during transport, reduced metabolism, and the elimination of xenobiotics, among other reasons.[18] Accordingly, for example, the quality regulations of Norwegian food laws indicate that the fish should be starved to empty the gut before harvesting to ensure proper hygiene for further processing. Among the benefits of this practice include the reduction of physical activity, fighting among the fish related to the instinct to maintain dominance hierarchies, and stresses related to acute crowding and other factors during transportation.[19] The common current practice is to starve the fish for 3 to 4 days before harvest and, under low temperature conditions, the fish should be starved for at least 5 to 7 days

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[15] Kennish et al. (1985) cites Forlin L, Anderson T, Koivusaari U and Hansson T (1984). Influence of biological and environmental factors on hepatic steroid and xenobiotic metabolism in fish: Interaction with PCB and β-naphthoflavone. Mar. Environ. Res. 14: 47-58.

[16] Roubal WT, Collier K, Malins DC (1977). Accumulation and metabolism of carbon-14 labeled benzene, naphthalene, and anthracene by young coho salmon (Oncorhychus Kisutch). Arch. Environ. Contam. Toxicol. 5: 513-529.

[17] 2.5 µCi ^{14}C-benzene ÷ (198 µCi/mg benzene x 1 g bw) x 1000 g/kg = 12.6 mg benzene/kg bw; the body weight of fingerling Coho salmon was assumed to be similar to that reported by Luzzanna U, Hardy RW, Halver JR (1998). Dietary arginine requirement of fingerling coho salmon (Oncorhychus kisutch). Aquaculture 163: 137-150 (i.e. mean 0.9 ± 0.02 g S.E.M.).

[18] Waagbo R, JHorgensen SM, Timmerhaus G, Breck O, Olsvik PA (2017). Short-term starvation at low temperature prior to harvest does not impact the health and acute stress response of adult Atlantic salmon. Peer J 5:e3273; DOI 10.7717/peerj.3273: https://peerj.com/articles/3273.pdf.

[19] VKM (2008). Opinion of the Panel on Animals Health and Welfare of the Norwegian Scientific Committee for Food Safety: Transportation of fish within a closed system. VKM Report 2008: 23, 07/806-Final. 14 May 2008. 63 pp. (https://vkm.no/download/18.d44969415d027c43cf154e6/1500390477876/577c2a6603.pdf).

pre-harvest. Thus, if there were any residual n-hexane, cyclohexane, benzene or toluene from natural gas in salmonid food containing FeedKind®, it is reasonable to expect that none of these substances would remain in the bodies of the fish after 3 or more days of starvation prior to transport and slaughter.

Based on these published reports, we assumed, conservatively for our screening-level safety assessment calculations, that 0.066% (i.e. the percent of the administered radiolabel remaining in flesh after only 6 hours reported by Roubal *et al.* 1977) of the daily oral intake of benzene in salmonid food remains in the edible tissue of the fish when it is harvested and consumed. In addition, we assumed that other natural gas constituents are metabolized in the fish and/or by *M. capsulatus* to the same overall extent, based on the published reports of Kennish *et al.* (1985, 1988), Colby *et al.* (1977) and studies cited therein, which clearly demonstrated the capacity of fish liver enzymes and *M. Capsulatus* to metabolize these substances rapidly.

High-end exposures were estimated for human consumption of salmon and trout raised on diets containing 18% FeedKind® based on the highest calculated concentrations of natural gas constituents in the salmonid feed (**Table 4**). Additional assumptions included:

- Cumulative feed consumed by the target animal per weight of edible tissue (i.e. 1.77 and 2.14 kg feed/kg edible body weight for Atlantic salmon and trout, respectively)[20]
- 100% of the intake of each constituent from the feed accumulates in the edible fish tissue
- High chronic daily consumption of salmon or trout by humans is equal to the 90th percentile daily ingestion level of all finfish (i.e., 0.17 kg/day)[21]
- Body weight 70 kg[22]

Maximum estimated daily intakes (EDIs) of the constituents from the consumption of salmon and trout, based on these highly exaggerative assumptions, are presented in **Table 5**.[23]

Table 5. Maximum Estimated Daily Intake (EDI) of Natural Gas Constituents from Fish Consumption

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[20] *See* Table 2 in Fry JP, Mailloux NA, Love DC, Milli MC, Cao L (2018). Feed conversion efficiency in aquaculture: do we measure it correctly? Environ. Res. Lett. 13: 024017: https://iopscience.iop.org/article/10.1088/1748-9326/aaa273/pdf.

[21] *See* Table 2.055 in Smiciklas-Wright H, Mitchell DC, Mickle SJ, Cook AJ, Goldman JD (2002). USDA 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII 1994-1996).

[22] NRC (2005) specifies an MRL of 0.0003 mg Hg/kg bw/day for a 70-kg person

[23] For example, [7.27 ppm benzene in salmonid feed x 1.77 kg feed/kg edible salmon tissue x 0.066% benzene oral intake remaining in edible tissue x 0.17 kg salmon/day]/70 kg bw = 2.06 x 10^{-5} mg benzene/kg bw/day.

Constituent(s)	Maximum Concentration in Edible Salmon Tissue (ppm)	Maximum Concentration in Edible Trout Tissue (ppm)	Maximum EDI from Salmon Consumption (mg/kg bw/day)	Maximum EDI from Trout Consumption (mg/kg bw/day)
Hexanes	0.022	0.027	5.37×10^{-5}	6.49×10^{-5}
Cyclohexane	0.003	0.004	7.45×10^{-4}	9.00×10^{-4}
Benzene	0.008	0.010	2.06×10^{-5}	2.49×10^{-5}
Toluene	0.200	0.242	4.87×10^{-4}	5.88×10^{-4}

Toxicity reference values for risk assessment have been developed by US EPA Integrated Risk Information System (IRIS) Program for all of the substances assumed to remain in the edible tissue of salmonids fed FeedKind® at the highest use level in fish food (i.e. 18%), including hexane, cyclohexane, benzene and toluene. These toxicity values include a cancer slope factor (CSF)[24] for benzene, non-cancer reference doses (RfDs)[25] for chronic oral exposures to benzene and toluene, and a reference concentrations (RfCs) for chronic inhalation exposure to n-hexane and cyclohexane. As noted above, oral exposures to n-hexane and cyclohexane, unlike inhalation exposures to these substances, have not been shown to be associated with oral toxicity or developmental toxicity, respectively. However, we derived a chronic oral RfDs from the inhalation RfCs for n-hexane and cyclohexane in an abundance of caution in this screening level safety assessment.[26] The toxicity values used in this assessment are presented in **Table 6.**

Table 6. Toxicity Values for Selected Natural Gas Constituents

Constituent(s)	Chronic Oral RfD (mg/kg bw/day)	Cancer Slope Factor $(mg/kg\ bw/day)^{-1}$	Critical Effect(s)	Reference
n-Hexane	0.2[27]	ND[28]	Peripheral neuropathy	n-Hexane | IRIS | US EPA

[24] Oral Slope Factor: "An upper bound, approximating a 95% confidence limit, on the increased cancer risk from a lifetime oral exposure to an agent. This estimate, usually expressed in units of proportion (of a population) affected per mg/kg-day, is generally reserved for use in the low-dose region of the dose-response relationship, that is, for exposures corresponding to risks less than 1 in 100." IRIS Glossary | Integrated Risk Information System | US EPA."

[25] Reference dose: An "estimate, with uncertainty spanning perhaps an order of magnitude, of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime"; IRIS Glossary | Integrated Risk Information System | US EPA.

[26] For approach to converting RfCs to RfD see, for example, https://rais.ornl.gov/tutorials/toxvals.html#2.4%20Derivation%20of%20Inhalation%20RfDs%20and%20Slope%20Factors.

[27] n-Hexane RfD = 0.7 mg/m^3 RfC x 20 m^3/day ÷ 70 kg bw = 0.2 mg/kg bw/day.

[28] ND = not determined; there are no data indicating an association between cancer and human exposure to these substances.

Cyclohexane	1.7[29]	ND	Reduced pup weights in 2-generation rat developmental toxicity test	Cyclohexane (CASRN 110-82-7) | IRIS | US EPA
Benzene	4×10^{-3}	0.015	Decreased lymphocyte count; leukemia	Benzene (CASRN 71-43-2) | IRIS | US EPA
Toluene	0.08	ND	Increased kidney weight in rats	Toluene (CASRN 108-88-3) | IRIS | US EPA

The toxicity reference values presented in **Table 7** were used to calculate the upper bound cancer risk estimate for benzene and hazard quotients (HQs)[30] for potential non-cancer effects presented in **Table 7**.

Table 7. Worst-Case Risk Estimates for EDI of Potential Gas Constituents through Fish Consumption

	Salmon consumption		**Trout Consumption**	
Constituent(s)	**HQ for Potential Non-Cancer Effects (unitless)**	**Upper-Bound Cancer Risk Estimate (unitless)**	**HQ for Potential Non-Cancer Effects (unitless)**	**Upper-Bound Cancer Risk Estimate (unitless)**
Hexanes	$2.68 \text{ X } 10^{-4}$	NA	$3.25 \text{ X } 10^{-4}$	NA
Cyclohexane	$24.34 \text{ X } 10^{-6}$	NA	$5.25 \text{ X } 10^{-6}$	NA
Benzene	$5.16 \text{ X } 10^{-3}$	3.09×10^{-7}	$6.230 \text{ X } 10^{-3}$	3.74×10^{-7}
Toluene	$6.08 \text{ X } 10^{-3}$	NA	$5.88 \text{ X } 10^{-3}$	NA

Table 8 presents the corresponding central tendency risk estimates calculated by substituting the highest concentration by the median concentration of each constituent of natural gas reported in Chao and Attari (1995).

Table 8. Central Tendency Risk Estimates for EDI of Gas Constituents through Fish Consumption

	Salmon consumption		**Trout Consumption**	
Constituent(s)	**HQ for Potential Non-Cancer Effects (unitless)**	**Upper-Bound Cancer Risk**	**HQ for Potential Non-Cancer Effects (unitless)**	**Upper-Bound Cancer Risk**

[29] Cyclohexane RfD = 6 mg/m^3 RfC x 20 m^3/day ÷ 70 kg bw = 1.7 mg/kg bw/day.

[30] Hazard quotient: the ratio of the potential exposure to a substance (i.e. the EDI) and the level at which no adverse effects are expected (i.e. the RfD).

		Estimate (unitless)		Estimate (unitless)
Hexanes	3.96×10^{-5}	NA	4.79×10^{-5}	NA
Cyclohexane	7.14×10^{-7}	NA	8.64×10^{-7}	NA
Benzene	7.68×10^{-5}	4.61×10^{-9}	9.29×10^{-5}	5.57×10^{-9}
Toluene	3.66×10^{-4}	NA	3.54×10^{-4}	NA

The results presented in **Table 7** and **Table 8** clearly show the upper bound cancer risk estimate for benzene is less than 10^{-6} (i.e. *de minimis*), and all HQs for all natural gas constituents would be orders of magnitude less than 1 even at the maximum concentrations of constituents reported in natural gas and exaggerative worst case exposure assumptions. Thus, there is no reasonable expectation of harm associated with the consumption of salmonids fed FeedKind® up to the highest use level in salmonid food (i.e. 18%).

The results of the safety assessment presented above also support the safety of the target animals, namely salmonid. This is because, in addition to the exaggerative exposure assumptions, the toxicity values used to estimate the non-cancer and cancer risks are at least 300-fold less than the no observed effect levels (NOAELs) or Benchmark Dose Low (BMDL = lower confidence limit of the BMD) for n-hexane, cyclohexane, and benzene and 3000-fold less than the BMDL for toluene from animal studies, which indicates that the margin of safety for the target animals is orders of magnitude greater than the margin of safety for the protection of human health.

As noted above, the natural gas available to users in the U.S. may contain a wide range of benzene, toluene, cyclohexane and hexanes concentrations, based on the survey of Chao and Attari (1995) and benzene and toluene concentrations based on the 3 examples representing the spectrum of natural gas products in the U.S. reported by Black and Veatch (2021). These values are presented in **Table 9** below.

Table 9. Range and Median of Constituent Concentrations Reported in Natural Gas in the US

	Chao and Attari (1995)		Black and Veatch (2021)	
Constituent(s)	**Range (ppmv)**	**Median(ppmv)**	**Range (ppmv)**	**Median (ppmv)**
Hexanes	<0.2 to 1156	170	NR[31]	NR
Cyclohexane	<0.2 to 146	24	NR	NR
Benzene	<0.2 to 471	7	44 to 470	230
Toluene	<0.1 to 100	6	5 to 100	18

In coordination with its suppliers, Calysta will monitor the natural gas received to its facility with gas chromatography to ensure that the benzene concentration does not exceed 40 ppmv. Further, in the site selection process for production facilities, Calysta will preferentially

[31] Black and Veatch (2021) presented data for "n-Hexane+", which covers all alkanes ≥C6 in size, and did not provide values specifically for hexanes or cyclohexane.

choose gas supplies and regional locations with reliably low contaminant levels. Calysta will not use natural gas that contains ≥40 ppmv benzene to produce FeedKind®. This approach will also ensure that the natural gas used to produce FeedKind® will also contain toluene and other constituents at the lower end of the respective ranges reported for these compounds in natural gas in the U.S., and substantially lower than the 40 ppmv threshold for benzene because:

- The levels of compounds like toluene are characteristically lower than the corresponding benzene concentration in the natural gas.
- The predominant method in North America for the removal of aromatics and natural gas liquids (NGLs) from natural gas is cryogenic expansion. The efficiency of this removal process is largely a function of the boiling point of the respective gases. As benzene has the lowest boiling point of the targeted aromatic contaminants in natural gas, a maximum limit on benzene will in effect limit toluene and cyclohexane as well.

Ensuring that the concentration of benzene does not exceed 40 ppmv ensures that the natural gas used to produce FeedKind® contains no more than approximately 8.5% of the maximum concentration of benzene reported in the natural gas surveys, and that FeedKind® cannot possibly contain more than approximately 3.4 ppm benzene.[32] No benzene or related compounds are expected to be present in FeedKind®. However, it is clear that the worst-case risk estimates presented in **Table 7** for benzene and the other natural gas constituents are overestimated by at least an additional factor of 10.

In an abundance of caution, we calculated risk estimates assuming that the concentrations of the constituents in the natural gas used to produce FeedKind® is 8.5% of the respective maximum concentrations reported in the natural gas, using the same approach as above for non-cancer endpoints except that we assumed that none of the constituents is metabolized in the fermenter or detoxified by the fish. The results are presented in **Table 10**.

Table 10. Worst-Case Risk Estimates for Potential Gas Constituents Assuming No Metabolism in the Fermenter or Detoxification in Fish

	Salmon	**Trout**
Constituent(s)	**HQ for Potential Non-Cancer Effects (unitless)**	**HQ for Potential Non-Cancer Effects (unitless)**
Hexanes	0.0346	0.0418
Cyclohexane	0.000559	0.000676
Benzene	0.664	0.803
Toluene	0.783	0.758

[32] 40 ppmv benzene threshold ÷ 471 ppmv benzene maximum reported x 100 = 8.49%; 40.4 ppm benzene in FeedKind® from 471 ppmv maximum reported benzene natural gas x 8.49% = 3.43 ppm maximum benzene concentration in FeedKind®.

All of the HQs for these constituents are less than 1, indicating that there is no reasonable expectation of harm from the high-end consumption of salmon or trout fed food containing up to 18% FeedKind® manufactured using natural gas containing no more than 40 ppmv benzene.

In addition, we calculated risk estimates for the cancer endpoint for benzene based on the same assumptions, except that we assumed that 1%, rather than 99.934% (i.e. 100%-0.066%), of the dose was not effectively detoxified in the bodies of the fish. The resultant cancer risk estimates were 3.98×10^{-7} and 4.82×10^{-7} for salmon and trout consumption, respectively. Again, these risk estimates are *de minimis*.

Overall, the results of these calculations, based on the exaggerative exposure assumptions and the safety factors used to calculate potential lifetime human health risks, show that there is no reasonable expectation of harm to the target animals or to consumers from the intended use of FeedKind® in fish food.

We do not have data to characterize the constituents of the natural gas used to manufacture the BioProtein® that was tested in the animal studies. However, it is clear from the analysis presented above that maintaining a threshold of 40 ppmv benzene in the natural gas used to manufacture FeedKind® used as intended presents no safety concern to salmon or to consumers.

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Natural gas production often generates hydrocarbon streams containing trace levels of mercury (Hg), predominantly elemental mercury (Hg^0) in the gas phase.[1] For example, Corvivni et al. (2002) reported Hg concentrations ranging from below detection limits up to 120 µg/Nm^3.[2] However, the Hg that may be present must be removed from natural gas to be transported by pipeline to protect downstream aluminum (Al) heat exchangers from catastrophic mechanical failure and gas leakage caused by the amalgamation of Hg with the Al of the exchangers over time.[3] As well, Hg must be removed from natural gas to prevent catalyst deactivation in the production of ethylene from the ethane or propane of the natural gas, for example. Chao and Attari (1993) did not detect Hg in pipeline natural gas in a survey of gas samples across the gas distribution system in the US using a measurement method for which the detection limits for Hg ranged from 0.02 to 0.2 µg/Nm^3.[4] Current industry practices reduce Hg concentrations to < 0.01 µg/Nm^3.

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[1] Porcheron F, Barthelet K, Schweitzer JM, Daudin A (2012). Mercury traces removal from natural gas: Optimization of guard bed adsorption properties. Conference paper presented at the 2012 American Institute of Chemical Engineers (AIChE) Annual Meeting, Environmental Applications of Adsorption I: Gas Phase, 1 November 2012: https://www.aiche.org/conferences/aiche-annual-meeting/2012/proceeding/paper/632e-mercury-traces-removal-natural-gas-optimization-guard-bed-adsorption-properties.

[2] Corvini G, Stiltner J, Clark K (2002). Mercury removal from natural gas and liquid streams. UOP LLC, Houston TX; https://web.archive.org/web/20110101194809/http:/www.uop.com/objects/87MercuryRemoval.pdf; Nm^3 = volume in m^3 at normal temperature and pressure; the International Standard Metric Conditions for natural gas and similar fluids are 288.15 K (15.00 °C; 59.00 °F) and 101.325 kPa; https://www.iso.org/standard/20461.html.

[3] *See also* Aly MAEl E, Mahgoub IS, Nabawi M, Ahmed MAA (2008). Mercury monitoring and removal at gas-processing facilities: case study of Salam gas plant. SPE Proj. Facilit. Construct. 3(1): 1-9: https://www.researchgate.net/publication/250091182_Mercury_Monitoring_and_Removal_at_Gas-Processing_Facilities_Case_Study_of_Salam_Gas_Plant; Porcheron F, Barthelet K, Schweitzer JM, Daudin A (2012). Mercury traces removal from natural gas: Optimization of guard bed adsorption properties. Conference paper presented at the 2012 American Institute of Chemical Engineers (AIChE) Annual Meeting, Environmental Applications of Adsorption I: Gas Phase, 1 November 2012: https://www.aiche.org/conferences/aiche-annual-meeting/2012/proceeding/paper/632e-mercury-traces-removal-natural-gas-optimization-guard-bed-adsorption-properties.

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Mercury vapor (Hg^0), inorganic mercury compounds (Hg^{2+}), and methylmercury (MeHg) are well studied environmental toxicants.[5] Atmospheric Hg^0 vapor is derived from natural degassing of the earth crust and through volcanic eruptions as well as from anthropogenic sources.[6] Eventually, atmospheric Hg^0 is oxidized to water-soluble inorganic forms (Hg^{2+}) and returned to the surface in rainwater, from which Hg^{2+} can be reduced back to Hg^0 and returned to the atmosphere, or the Hg^{2+} may be methylated by microorganisms to produce MeHg in the sediments of freshwater and saltwater bodies. The MeHg produced in this way can enter the food chain starting with plankton, and then bioaccumulate in the food chain through herbivorous fish up to carnivorous fish and sea mammals. The bioaccumulation of Hg can result in MeHg levels in the tissues of fish and mammals at the top of the food chain that are from 1800 up to 80,000 times greater than the corresponding Hg concentrations in the water in which these animals live and feed. In turn, the bioaccumulation of MeHg can result in human exposures through the consumption of fish, especially fish at the top of the food chain (e.g., swordfish and shark) in which MeHg tissue concentrations are typically substantially greater than the concentrations in fish at lower levels of the food chain (e.g., salmon and trout).[7]

MeHg and other organomercurial compounds are generally recognized to be more potent toxicants than inorganic Hg compounds in fish and mammals, including humans, especially for exposures that occur during early life stages of development. NRC (2005) noted that salmon and poultry tolerate chronic exposures to MeHg at up to 1 mg Hg/kg diet, and the NRC (1980) established a dietary MeHg level of 2 mg Hg/kg diet as safe for swine and ruminants.[8] On a per-kg-body-weight (bw) basis, nonreproducing rodents and cats tolerated chronic MeHg exposure to 0.1 mg/kg bw/day MeHg (i.e. 100 µg/kg bw/day). During reproduction, rodents, nonhuman primates and cats tolerated chronic MeHg exposure to 5 µg Hg/kg bw/day in all studies reviewed. For human exposure to Hg in fish, NRC (2005) set a maximum tolerable level

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[5] For reviews, see: Goyer RA and Clarkson TW (2001). Toxic Effects of Metals: Iron (Fe). Chapter 23 In: Casarett and Doull's Toxicology: The Basic Science of Poisons. 6th Edition. Klaassen CD Editor. McGraw-Hill. Pp. 834-837; Tokar EJ, Boyd WA, Freedman JH, Waalkes MP (2013). Toxic Effects of Metals: Iron (Fe). Chapter 23 In: Casarett and Doull's Toxicology: The Basic Science of Poisons. 8th Edition. Klaassen CD Editor. McGraw-Hill. Pp. 996-999.

[6] For reviews, see id.

[7] For example, see: US FDA (undated). Mercury levels in commercial fish and shellfish (1990-2012): https://www.fda.gov/food/metals/mercury-levels-commercial-fish-and-shellfish-1990-2012; US FDA (2019). Technical information on development of FDA/EPA advice about eating fish for women who are or might become pregnant, breastfeeding mothers, and young children: https://www.fda.gov/food/metals/technical-information-development-fdaepa-advice-about-eating-fish-women-who-are-or-might-become.

[8] National Research council (NRC) (2005). Mercury. Chapter 20 in: Mineral tolerance of animals. Committee on Minerals and toxic Substances in diets and water for Animals, Board on agriculture and Natural resources, Division on Earth and Life Studies, Second Revised Edition, pp. 276-283.

(MRL)[9] of 0.3 µg Hg/kg bw/day for human exposure based on the potential for effects on the neurodevelopment of children exposed in utero to MeHg from maternal fish ingestion.[10]

The specification for Hg in pipeline gas used in the production of FeedKind® is 0.02 µg/Nm3 maximum, and Calysta will not accept or use pipeline natural gas that is not certified to contain Hg ≤ 0.02 µg/Nm^3 for manufacturing this product.

Exaggerative estimates of exposures to mercury were calculatedassuming that salmon or trout are raised on feed containing the maximum proposed use level of FeedKind® (i.e. 18%) and other conservative assumptions. FeedKind® is manufactured in 12-week fermentation cycles using pipeline natural gas containing mercury at the maximum concentration defined by specification in this GRASN (i.e. 0.02 µg/Nm^3). Our estimates were calculated based on an example production scenario in which FeedKind® is synthesized continuously by the bacterial consortium in a fermenter during a 12-week cycle, and fractions of the biomass and associated culture medium are constantly conveyed to a centrifuge in which the biomass is separated from the culture medium. The biomass is processed further downstream to produce FeedKind®. The culture medium that is separated from the biomass is recycled to the fermenter. The fermenter and all downstream manufacturing equipment will be emptied and cleaned at the end of each 12-week cycle and prepared to receive fresh bacterial culture and medium at the start of the next 12-week cycle.

Approximately 5.5 x 10^6 Nm^3 natural gas will be consumed during each 12-week cycle to produce 2,308 tons (2.1 x 10^6 kg) dry biomass (i.e. FeedKind®) during each 12-week cycle.[11] If the concentration of Hg is assumed to be constant at the maximum 0.02 µg/Nm^3 in the pipeline gas during production, then approximately 109 mg Hg will have been delivered to the reactor during the 12-week cycle.[12]

The Hg introduced into the reaction vessel from the natural gas will partition into two principal fractions of the bacterial culture, including the aqueous growth medium (i.e. the medium fraction) and the bacterial cells (i.e. the biomass fraction) during fermentation. Most of the Hg in the natural gas will be inorganic. However, the bacterial cells will likely convert at least some of the inorganic Hg to MeHg, which is much more toxic and hydrophobic than the

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9 MRL = maximum tolerable level = the dose that can be ingested for a lifetime without significant risk of adverse effects; the MRL for MeHg assumes 70-kg maternal BW.

10 In comparison, the UN FAO and WHO set a maximum mercury intake of 0.23 µg/kg bw/day to protect the developing fetus and ATSDR recommended that pregnant women do not consume fish containing more than 250 µg Hg/kg; NRC (2005) noted that these agencies "stressed that public health authorities should keep in mind that fish play a key role in meeting nutritional needs in many countries"; US FDA's "action level" for MeHg is 50 µg/kg for fish in interstate commerce.

11 10,000 tons FeedKind® produced per year; (10,000 tons/year ÷ 52 weeks/year) x 12 weeks/cycle = 2,308 tons FeedKind® produced per cycle; 2,365 Nm^3 natural gas consumed per ton; 2,308 tons FeedKind®/cycle x 2,365 Nm^3 natural gas/ton FeedKind® = 5.4584 x 10^6 Nm^3 natural gas/cycle; 2,308 tons FeedKind® x 907.185 kg/ton = 2.0938 x 10^6 kg FeedKind®

12 5.45842 x 10^6 Nm^3 natural gas/cycle x 0.02 µg Hg/Nm^3 natural gas = 1.09 x 10^5 µg Hg/cycle = 1.1097 x 10^5 µg Hg/cycle ÷ 1000 µg/mg = 109 mg Hg/cycle.

inorganic forms of Hg. The much greater bioconcentration factors (BCFs) and bioaccumulation factors (BAFs) typically reported for MeHg, compared with inorganic Hg compounds, is attributable to the substantially greater lipophilicity of MeHg.[13] For example, US EPA (1995) estimated BCFs of 52,175 kg/l and 2,998 kg/l for MeHg and inorganic Hg, respectively, based on the results of laboratory tests with MeHg and highly soluble forms of inorganic Hg.[14] These BCFs are within the 1800 to 80,000 range generally reported for Hg in carnivorous fish and sea mammals at the top of the food chain.[15]

As noted above, the fermenter will receive approximately 109 mg Hg during the production of (b) (4) kg dry FeedKind® in each 12-week cycle if the Hg concentration in the natural gas is always equal to the specified maximum of 0.02 µg/Nm^3 throughout the cycle. FeeedKind® will be produced in a re-circulating system in which the bacterial culture is continuously harvested, the harvested culture is centrifuged to separate the wet biomass from most of the medium, the wet biomass fraction is spray dried to produce FeedKind®, and the medium fraction is returned to the fermenter.

For the following calculations, the concentration of wet biomass in the harvested culture is assumed to be 2 g/100 ml (i.e. 2%) and the wet biomass production rate is (b) (4)r.[16] It follows that the concentration of medium in the harvested culture will be 98 g/100 ml (i.e. 98%).[17] The concentration of wet biomass in the harvested culture will increase from 2% to 35% through centrifugation and evaporation. Thus, 96.2% of the medium in the harvested culture will be returned to the fermenter, assuming conservatively and for simplicity, that the concentration to 35% wet biomass is achieved solely through centrifugation and that 100% of the separated medium is returned to the fermenter.[18] The calculations demonstrate a worst-case conservative

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13 Bioconcentration Factor (BCF): ratio of the concentration of a substance in an organism to the aqueous concentration as a result of direct uptake from the water; Bioaccumulation factor (BAF): ratio of the concentration of the substance in an organism to the aqueous concentration as the result of uptake from all exposure routes, including diet; BCFs and BAFs are often expressed as the ratio of mg of chemical per kg of organism to mg of chemical per liter of water (i.e. l/kg).

14 *See* New York State (1998). Human Health Fact Sheet: Ambient Water Quality Value Based on Human Consumption of Fish. March 12. 1998, https://www.epa.gov/sites/production/files/2015-06/documents/ny_hh_202_f_03121998.pdf; US EPA (1995). Great Lakes water Quality Initiative technical Support Document for the Procedure to Determine Bioaccumulation Factors. Office of Water 4301. EPA-820-B95-005.

15 Tokar EJ, Boyd WA, Freedman JH, Waalkes MP (2013). Toxic Effects of Metals: Iron (Fe). Chapter 23 In: Casarett and Doull's Toxicology: The Basic Science of Poisons. 8th Edition. Klaassen CD Editor. McGraw-Hill. p. 997.

16 (b) (4)

17 100% culture = 98% medium + 2% wet biomass.

18 For example, every 100 ml culture harvested will contain 2 g wet biomass and 98 ml medium before centrifugation. After centrifugation, the separated biomass fraction will contain

production scenario, as evidenced by the fact that only approximately 85%, rather than 96.2%, of the culture medium will be returned to the reactor after centrifugation and 15% will remain with the separated biomass to be concentrated through evaporation and spray drying to produce FeedKind®.

Based on these assumptions, the increase in the Hg concentration in the wet biomass (i.e. µg Hg/kg wet biomass) with the time of harvest (hours) over the 12-week cycle follows an exponential growth curve of the following form (correlation coefficient = 1.00):[19]

$$y = a(1-\exp(-bx)$$

where,

- y = concentration of Hg in wet biomass produced (µg Hg/kg)
- a = curve-fitting coefficient
- b = curve-fitting coefficient
- x = time of harvest (cumulative hours after cycle initiation)

These curves rise rapidly over the first few hours of each 12-week cycle, depending on the BCF assumed for Hg, and then much more slowly over the remaining hours of the 12-week cycle as the system approaches a dynamic equilibrium between the Hg that continues to enter the fermenter with the pipeline gas and the Hg that continues to be removed from the fermenter with the harvested wet biomass. **Table 13** presents the maximum concentrations of Hg in the wet biomass and dry biomass (i.e. FeedKind®) calculated assuming 1800 and 80,000 as the BCF for Hg and 2016 hours total cycle time (i.e. 12 weeks).

Table 13. Maximum Hg Concentrations in Wet Biomass and in FeedKind®

BCF	Coefficient a	Coefficient b	Hg Concentration in Wet Biomass (µg/kg)	Hg Concentration in FeedKind® (µg/kg)[20]
1800	1.041×10^{-2}	6.683×10^{-1}	1.041×10^{-2}	5.205×10^{-2}
80,000	1.042×10^{-2}	6.937×10^{-1}	1.042×10^{-2}	5.212×10^{-2}

Thus, the highest concentration of Hg, which will be in the last kg of 2.1×10^6 kilograms of FeedKind® produced during the 12-week production cycle, is approximately 0.052 µg/kg, assuming, conservatively, that the BCF for Hg in the fermenter is the highest BCF reported for

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[2 g wet biomass ÷ (3.71 ml medium + 2 g wet biomass)] x 100 = 35% wet biomass; it follows that the volume of the harvested medium returned to the fermenter will be (100 ml harvested culture – 5.71 separated culture) = 94.29 ml, which is (94.29 ml returned medium ÷ 98 ml harvested medium) x 100 = 96.2% of the harvested medium returned to the fermenter.

[19] Curve fitted using CurveExpert Professional (v.2.6.5); $5.79 \times 10^{-2} \times (1-\exp(-2.3 \times 2.1 \times 10^6) = 5.79 \times 10^{-2}$

[20] Hg concentration in FeedKind® = Hg concentration in wet biomass ÷ 0.2, assuming conservatively that the cells contain, by volume, 20% and 80% dry biomass and water, respectively.

Hg in fish and sea mammals at the top of the food chain and that no Hg vapor escapes the fermenter, centrifuge, spray dryer, or other elements of the production system during the cycle.[21]

It follows that salmonid feed containing 18% FeedKind® will contain no more than 0.289 µg Hg/kg feed.[22] This value is 3460 times lower than the 1 mg Hg/kg diet tolerated by salmon exposed chronically to dietary MeHg.[23] Thus, the risks to salmonids from chronic exposure to any Hg from pipeline natural gas in FeedKind® at up to the maximum use levels in fish feeds (i.e. 18%) is virtually non-existent.

High-end MeHg exposures were estimated for human consumption of salmon and trout raised on diets containing 18% FeedKind®, and assuming that 100% of the Hg in FeedKind® is in the form of MeHg. Again, the estimates were based on the highest calculated Hg concentration in the salmonid feed (i.e. 0.289 µg/kg feed). Additional assumptions included:

- Cumulative feed consumed by the target animal per weight of edible tissue (i.e. 1.77 and 2.14 kg feed/kg edible body weight for Atlantic salmon and trout, respectively)[24]
- 100% of the Hg intake from the feed accumulates in the edible fish tissue
- High chronic daily consumption of salmon or trout by humans is equal to the 90th percentile daily ingestion level of all finfish (i.e., 0.17 kg/day)[25]
- Body weight 70 kg[26]

Based on these highly exaggerative assumptions, the estimated daily intake (EDI) of Hg is 0.0012 and 0.0015 µg/kg bw/day for salmon and trout, respectively.[27] NRC (2005) specified an MRL of 0.3 µg Hg/kg bw/day for the protection of human health, for a 70-kg person, based on the potential for neurodevelopmental effects in children exposed in utero to methylmercury from maternal fish ingestion. Thus, the EDI is 250 and 200 times less than the MRL for the consumption of salmon and trout, respectively.[28]

Thus, the risks to consumers from chronic exposure to any Hg from pipeline natural gas in FeedKind® is negligible or virtually non-existent as well, even assuming that 100% of the fish

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21 For comparison, the calculated maximum Hg concentrations in FeedKind® assuming BCF=1 and BCF=10 is 0.018 µg/kg and 0.044 µg/kg, respectively.

22 0.052 µg Hg/kg FeedKind® ÷ 0.18 kg FeedKind®/kg feed = 0.289 µg Hg/kg fish feed

23 1 mg Hg/kg diet x 1000 µg/mg ÷ 0.289 Hg/kg fish feed = 3460.

24 *See* Table 2 in Fry JP, Mailloux NA, Love DC, Milli MC, Cao L (2018). Feed conversion efficiency in aquaculture: do we measure it correctly? Environ. Res. Lett. 13: 024017: https://iopscience.iop.org/article/10.1088/1748-9326/aaa273/pdf.

25 *See* Table 2.055 in Smiciklas-Wright H, Mitchell DC, Mickle SJ, Cook AJ, Goldman JD (2002). USDA 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII 1994-1996).

26 NRC (2005) specifies an MRL of 0.0003 mg Hg/kg bw/day for a 70-kg person

27 For example, [0.052 µg Hg/kg FeedKind® ÷ 0.18 kg FeedKind®/kg feed x 2.14 kg feed/kg edible trout tissue x 0.17 kg trout/day]/70 kg bw = 0.0015 µg Hg/kg bw/day.

28 MOE = MRL/EDI; for salmon, 0.3 µg/kg bw/day ÷ 0.0012 µg/kg bw/day = 250; for trout, 0.3 µg/kg bw/day x ÷ 0.0012 µg/kg bw/day = 200.

consumed by high-end fish consumers are salmon or trout raised exclusively on salmonid feed containing the maximum level of FeedKind® (i.e. 18%), all of which was produced using pipeline natural gas invariably containing the maximum possible concentration of Hg (i.e. 0.02 $\mu g/Nm^3$).

(b) (4)
29/06/2020

Appendix 6

60% & 69% Nitric Acid Analysis for (b) (4)

Analysis	60%	69%	Units
Assay	60.4	69.3	% m/m
Residue after ignition	<50	<50	ppm
Colour	Clear	Slight straw	
Total halides	<1	<1	ppm
Aluminium	<0.10	0.11	ppm
Antimony	<0.10	<0.10	ppm
Arsenic	<0.10	<0.10	ppm
Barium	<0.10	<0.10	ppm
Beryllium	<0.10	<0.10	ppm
Bismuth	<0.10	<0.10	ppm
Boron	<0.10	<0.10	ppm
Cadmium	<0.10	<0.10	ppm
Calcium	0.34	0.30	ppm
Chloride	<0.10	<0.10	ppm
Chromium	0.24	0.59	ppm
Cobalt	<0.10	<0.10	ppm
Copper	<0.10	<0.10	ppm
Gallium	<0.10	<0.10	ppm
Germanium	<0.10	<0.10	ppm
Gold	<0.10	<0.10	ppm
Indium	<0.10	<0.10	ppm
Iron	<0.10	0.23	ppm
Lead	<0.10	<0.10	ppm
Lithium	<0.10	<0.10	ppm
Magnesium	0.21	0.17	ppm
Mercury (ppb)	<0.005	<0.005	ppb
Molybdenum	<0.10	<0.10	ppm
Nickel	0.14	0.29	ppm
Niobium	<0.10	<0.10	ppm
Phosphate	<0.10	<0.10	ppm
Platinum	<0.10	<0.10	ppm
Potassium	<0.10	<0.10	ppm
Silver	<0.10	<0.10	ppm
Sodium	0.25	<0.1	ppm
Strontium	<0.10	<0.10	ppm
Sulphate	<0.10	<0.10	ppm
Tantalum	<0.10	<0.10	ppm
Thallium	<0.10	<0.10	ppm
Tin	<0.10	<0.10	ppm
Titanium	0.11	<0.10	ppm
Vanadium	<0.10	<0.10	ppm
Zinc	<0.10	<0.10	ppm
Zirconium	<0.10	<0.10	ppm

.

BATCH NUMBER	Ash (g/100 g)	Percentile
TEES-005/24	(b) (4)	
TPP-009/04		
TPP-009/06		
TEES-004/18		
TPP-004/01		
TPP-009/05		
TPP-013/06		
TEES-004/12		
TEES-004/15		
TEES-004/17		
TPP-007/08		
TPP-013/02		
TEES-004/2		
TEES-004/59		
TPP-004/08		
TPP-004/09		
TPP-013/04		
TPP-013/05		
TEES-009/79		
TPP-004/02		
TPP-004/12		
TPP-004/13		
TEES-004/3		
TEES-009/102		
TPP-004/14		
TPP-013/07		
TEES-004/6		
TEES-009/74		
TPP-004/04		
TPP-009/01		
TPP-009/09		
TEES-004/19		
TEES-006/RESEARCH2	(b) (4)	
TEES-009/75		
TPP-004/03		
TEES-004/23		
TEES-004/4		
TEES-009/73		
TPP-004/01		
TPP-007/09		
TPP-013/03		
TEES-004/11		

Mean
Median
Std. Deviation

Total

(b) (4)

TEES-007/02b
TEES-009/36
TEES-009/37
TEES-009/93
TEES-009/101
TEES-005/01
TEES-009/34
TEES-009/87
TPP-007/01
TPP-007/05
TPP-007/07
TPP-009/08
TPP-013/08
TEES-004/10
TEES-004/16
TEES-004/35
TEES-004/39
TEES-004/40
TEES-004/49
TEES-009/1
TEES-009/33
TEES-009/42
TEES-009/76
TPP-004/11
TPP-007/06
TPP-007/10
TEES-004/13
TEES-004/45
TEES-004/5
TEES-004/56

TEES-006/RESEARCH1
TEES-009/38
TEES-009/40
TEES-009/68
TEES-009/84
TPP-007/03
TEES-004/47
TEES-004/48
TEES-004/53
TEES-004/55
TEES-004/58
TEES-007/01b
TEES-009/28
TEES-009/39
TEES-009/49
TEES-009/78

(b) (4)

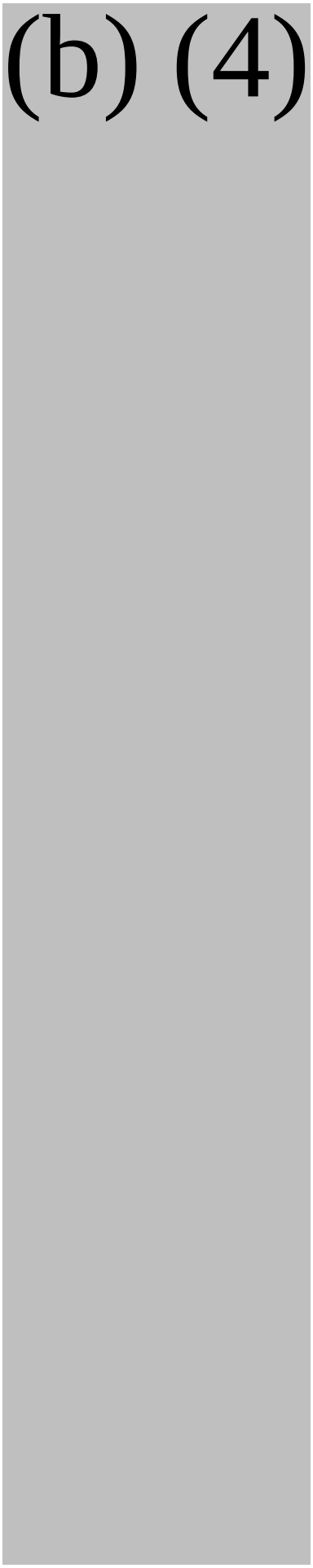
TEES-009/83
TPP-008/01
TEES-004/51
TEES-004/46
TEES-004/28
TEES-004/30
TEES-004/37
TEES-004/42
TEES-004/44
TEES-004/51
TEES-004/52
TEES-005/29
TEES-009/14
TEES-009/81
TEES-009/82
TEES-009/88
TPP-004/06
TEES-004/36
TEES-004/37
TEES-004/49
TEES-004/26
TEES-004/41
TEES-009/32
TEES-009/41
TEES-009/70
TEES-009/77
TEES-004/35
TEES-005/26
TEES-009/12
TEES-009/71
TEES-004/30
TEES-004/38
TEES-004/47
TEES-004/32
TEES-004/33
TEES-009/30
TEES-009/72
TEES-009/85
TPP-004/07
TPP-004/10
TEES-004/28
TEES-004/40
TEES-004/46
TPP-009/03
TEES-004/55
TEES-004/39
TEES-005/27

(b) (4)

TEES-005/28
TEES-009/29
TEES-009/63
TEES-009/80
TEES-009/86
TPP-007/02
TPP-007/04
TEES-004/31
TEES-005/25
TEES-009/15
TEES-009/21
TEES-009/31
TEES-009/19
TEES-009/20
TEES-009/26
TEES-004/52
TEES-004/42
TEES-005/23
TEES-004/24
TEES-004/34
TEES-004/54
TEES-004/9
TEES-004/53
TEES-004/48
TEES-004/27
TEES-004/38
TEES-009/52
TEES-009/58
TEES-009/59
TEES-004/43
TEES-004/54
TEES-004/31
TEES-009/61
TEES-009/62
TPP-004/05
TEES-005/03
TEES-009/64
TEES-009/67
TEES-004/25
TEES-009/16
TEES-009/25
TEES-009/51
TPP-011/01
TEES-005/41
TEES-005/42
TEES-009/17
TEES-009/24

(b) (4)

TEES-005/38
TEES-005/40
TEES-005/43
TEES-009/18
TEES-009/23
TPP-008/02
TEES-004/44
TEES-005/31
TEES-005/35
TEES-005/39
TEES-005/47
TEES-009/22
TEES-009/53
TPP-013/01
TEES-005/36
TEES-009/35
TEES-009/54
TEES-009/55
TEES-005/45
TEES-009/60
TEES-005/44
TEES-005/48
TEES-009/69
TEES-004/45
TEES-004/26
TEES-005/46
TEES-009/27
TEES-004/36
TEES-009/56
TEES-004/57
TEES-005/13
TEES-005/17
TEES-009/57
TEES-009/39
TEES-005/30
TEES-005/37
TEES-004/50
TEES-004/22
TEES-005/18
TEES-004/14
TEES-004/43
TEES-005/05
TEES-005/10
TEES-005/51
TEES-009/90
TEES-005/33
TEES-009/50



TPP-008/03
TEES-009/66
TEES-005/12
TEES-005/50
TEES-004/8
TEES-005/08
TEES-005/54
TEES-005/49
TEES-009/44
TEES-009/89
TEES-009/91
TEES-005/02
TEES-005/52
TEES-009/45
TEES-004/7
TEES-005/06
TEES-005/11
TEES-005/21
TEES-009/10
TEES-009/48
TEES-009/9
TEES-005/20
TEES-009/65
TEES-009/2
TEES-009/46
TEES-005/15
TEES-009/97
TEES-005/19
TEES-005/53
TEES-005/22
TEES-009/43
TEES-009/94
TEES-004/56
TEES-004/50
TPP-009/02
TEES-004/41
TEES-004/29
TEES-009/11
TEES-009/47
TEES-009/95
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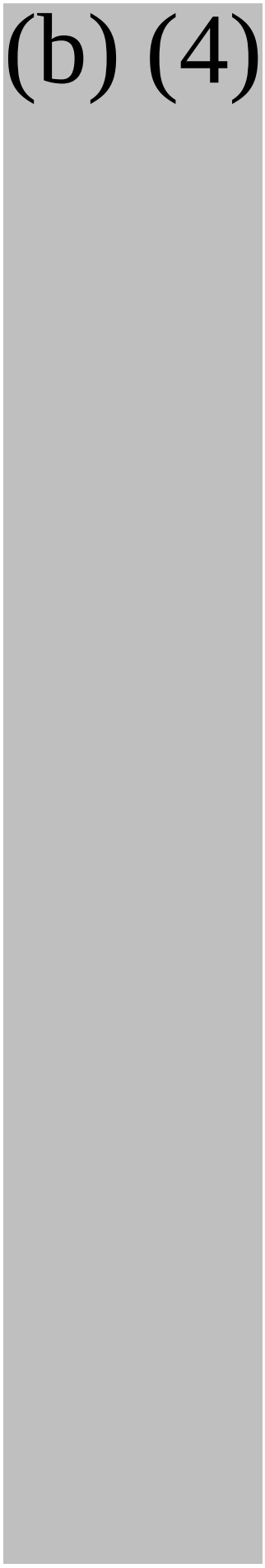
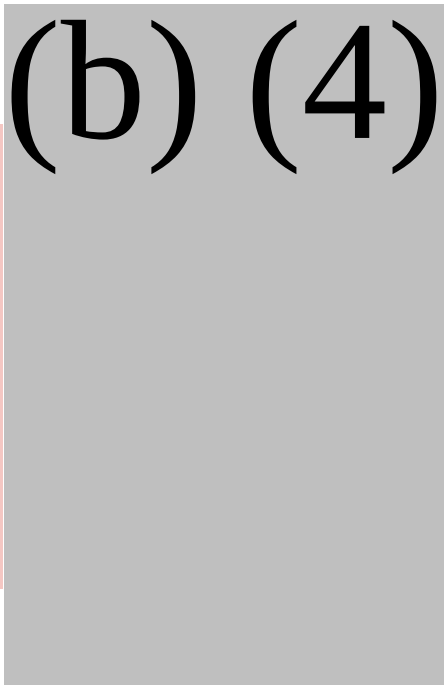
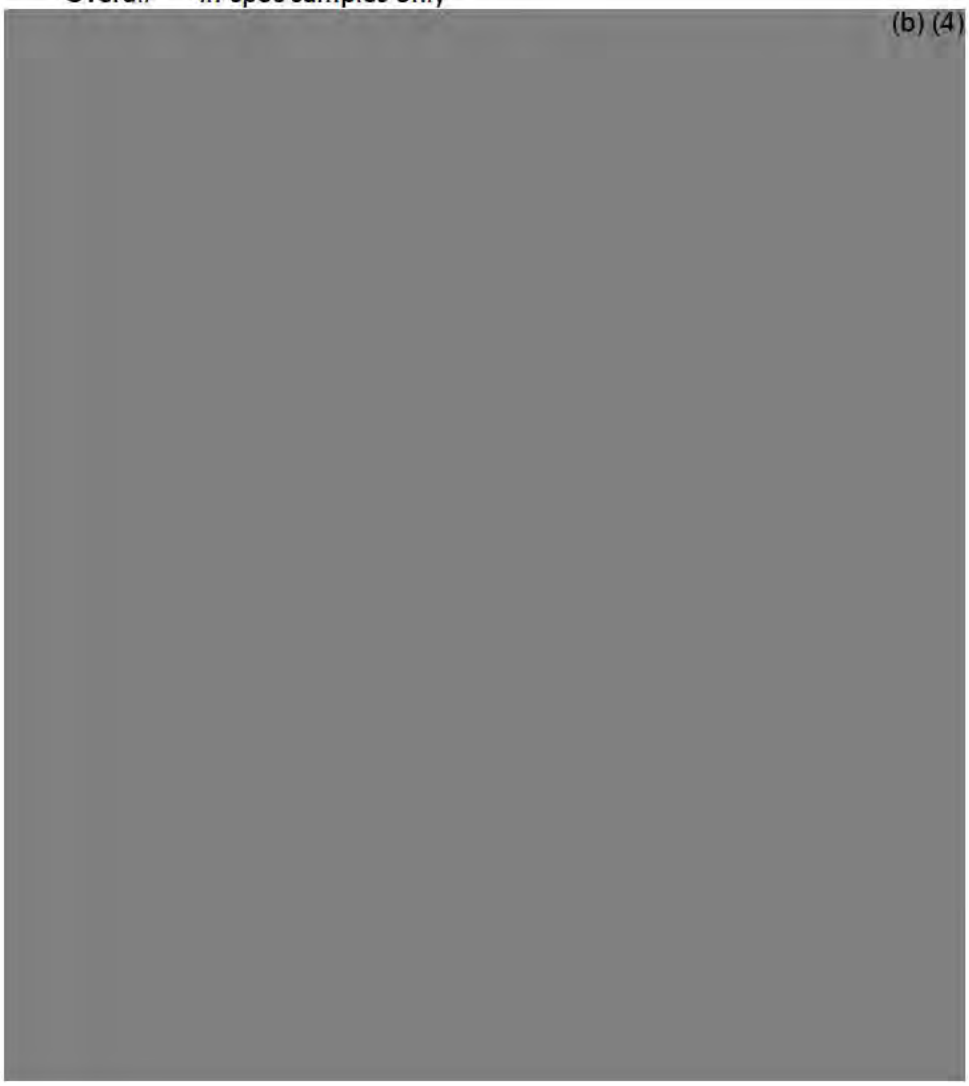
TEES-009/7
TEES-009/98
TEES-009/12
TEES-004/59
TEES-009/92



TEES-009/101
TEES-005/32
TEES-005/34
TEES-009/99
TEES-009/44
TEES-009/45
TEES-009/43
TEES-009/100
TEES-009/3
TEES-009/4
TEES-009/6
TEES-009/5
TEES-009/8
TEES-009/13



Overall In-spec samples only



(b) (4)

Method Summary

SOP Title :

SOP Number :

Introduction:

Principle:

(b) (4)

Accreditation Status:

(b) (4)

(b) (4)

(b) (4)

Method Validation Parameters:

(b) (4)

Reporting Format:

References:

Issue Date:

Review date:

(b) (4)

Appendix 3

Nickel by ICP-MS Validation Summary:

Full Validation of method S1172 – Heavy Metals by ICP-MS was carried out as part of the submission for (b) (4) accredited metals. Additional validation data for Nickel (not (b) (4) accredited) is shown below:

Spike Recovery:

A 10ppm spike solution was added to customer samples to evaluate recovery. Five samples were analysed unspiked, to quantify background levels, and spiked to evaluate recovery. The results are shown below.

Sample	Result(ppm) Ni 60		% Recovery
1	(b) (4)		
1			
2			
2			
3			
3			
4			
4			
5			
5			

As shown above all samples demonstrated recovery within the range of 100% +/- 10% which is considered satisfactory for this methodology.

Reproducibility:

In-house control samples used for other metals were used to assess reproducibility in feed and premix matrices. The results are shown below:

Feed Control		**Premix Control**	
Replicate	**Ni concentration (ppm)**	**Replicate**	**Ni concentration (ppm)**
1	(b) (4)		
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
Mean			
SD			
CoV (%)			

Results presented above show CoVs of 4.506% and 3.781% respectively for feed and premix matrices. These results demonstrate satisfactory reproducibility for Nickel under the analytical conditions specified in SOP S1172.

Appendix 8

(b) (4)ytical Methods – Summary.

(b) (4) has a number of ISO 178025:2017 accredited methods. The accreditation body that has assessed and granted accreditation for these methods is the United Kingdom Accreditation Service which is the sole national accreditation body recognised by the British government to assess the competence of organisations that provide certification, testing, inspection, and calibration services.

All accredited methods go through a validation process as described in our validation Standard Operating Procedure (SOP) S0006 – Validation of Analytical Methods and Calculation of Uncertainties.

The below excerpt from this SOP describes the validation process:-

THE TEST METHOD VALIDATION PROTOCOL

The Test Method Validation Protocol is an approved documented plan stating how the validation study will be conducted, including test parameters, product characteristics and sample preparation, test equipment, test regime, sampling plans, test procedure, data reporting and decision points on what constitutes acceptable test results.

EVALUATION OF ANALYTICAL METHODS

Two steps are required in the evaluation of an analytical method for validation:

Step 1: Determine the classification of the method by assigning the method to one or more of the following categories:

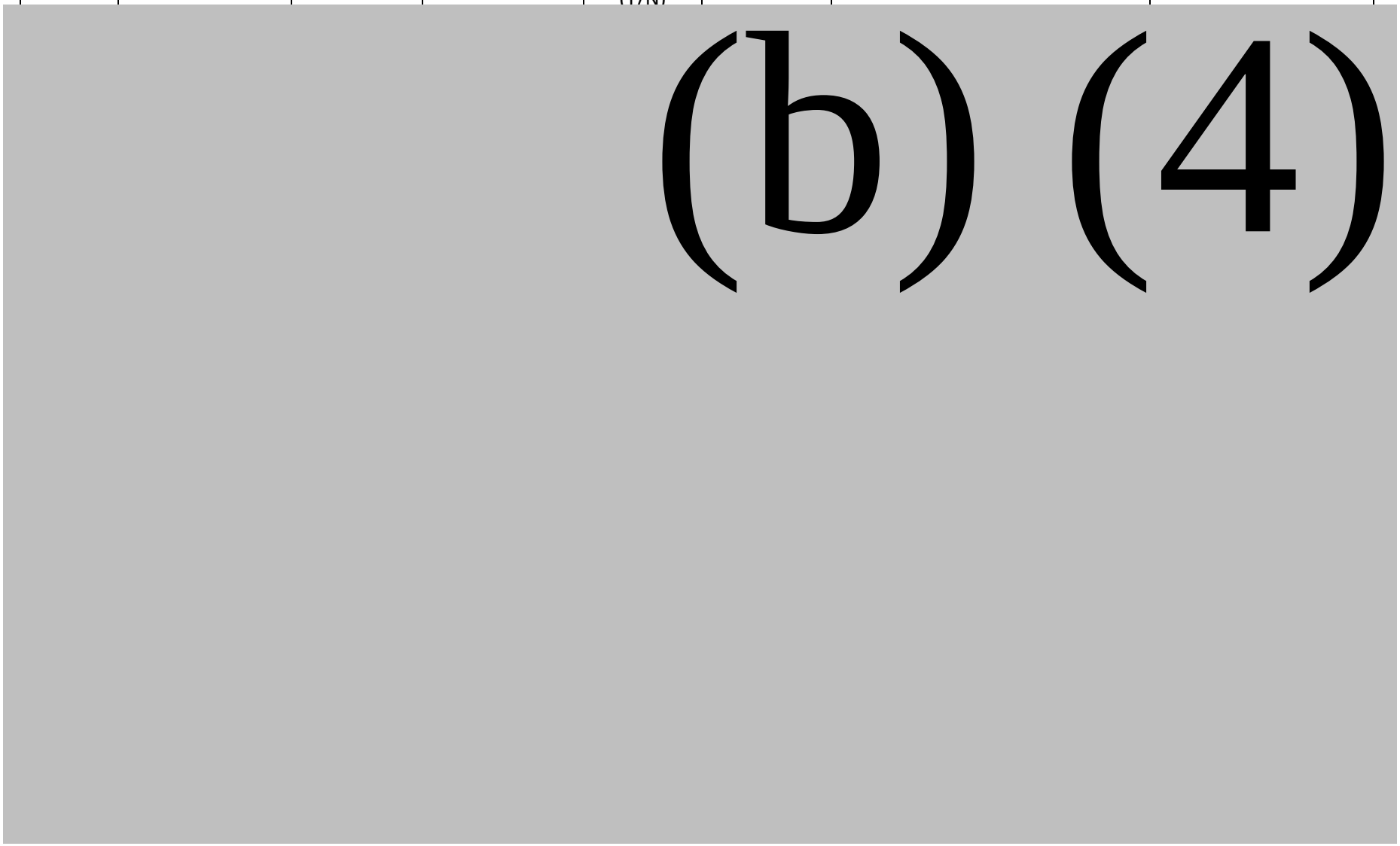
- *Qualitative (identification) Test*
- *Quantitative measurement for impurity content*
- *Limit tests for impurities*
- *Qualitative tests (analyte quantification)*
- *Physical tests*

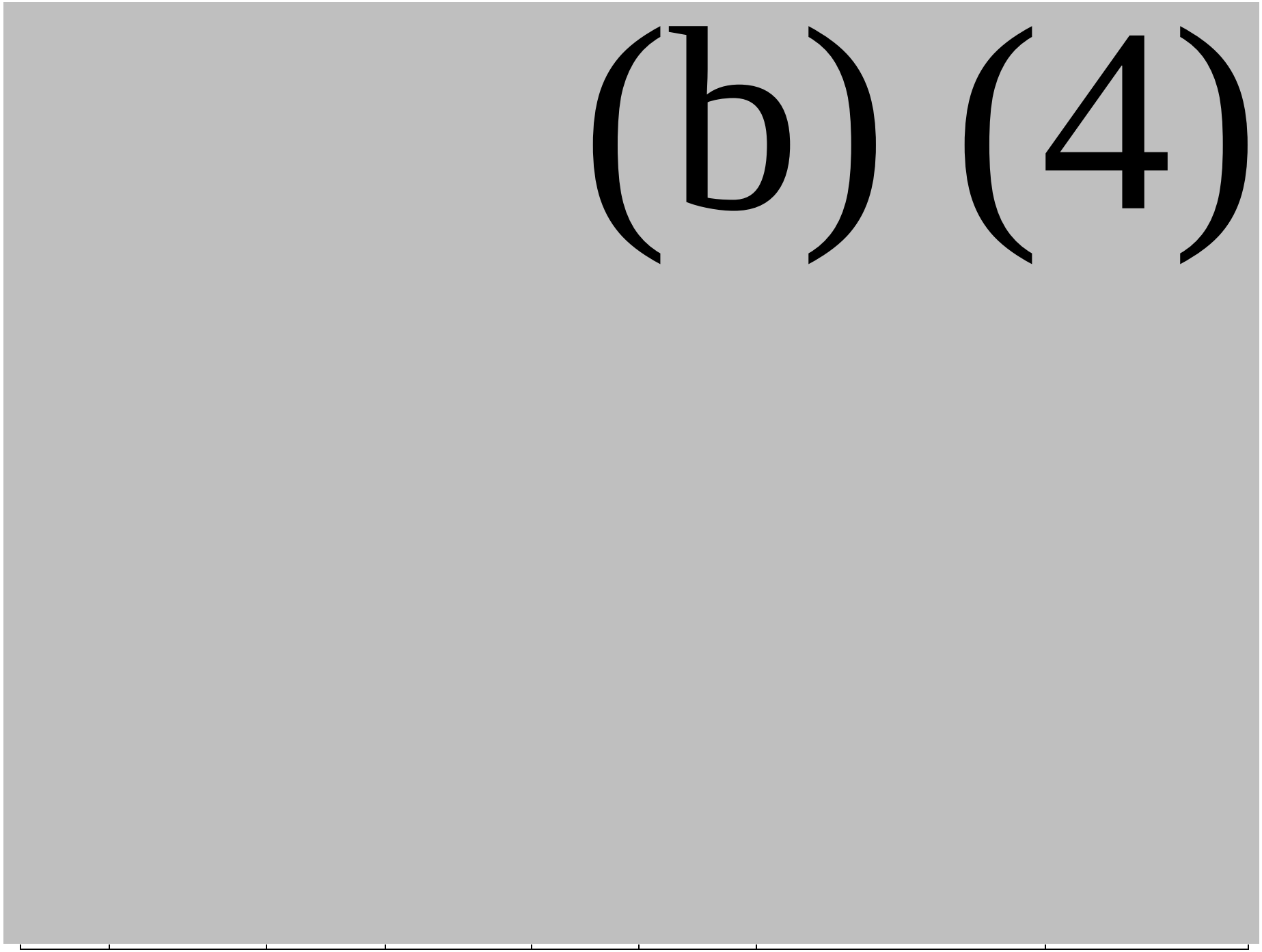
Step 2: Determine the characteristics of the method that require considering. The following characteristics are typically evaluated during validation but note that this is not an exhaustive list:

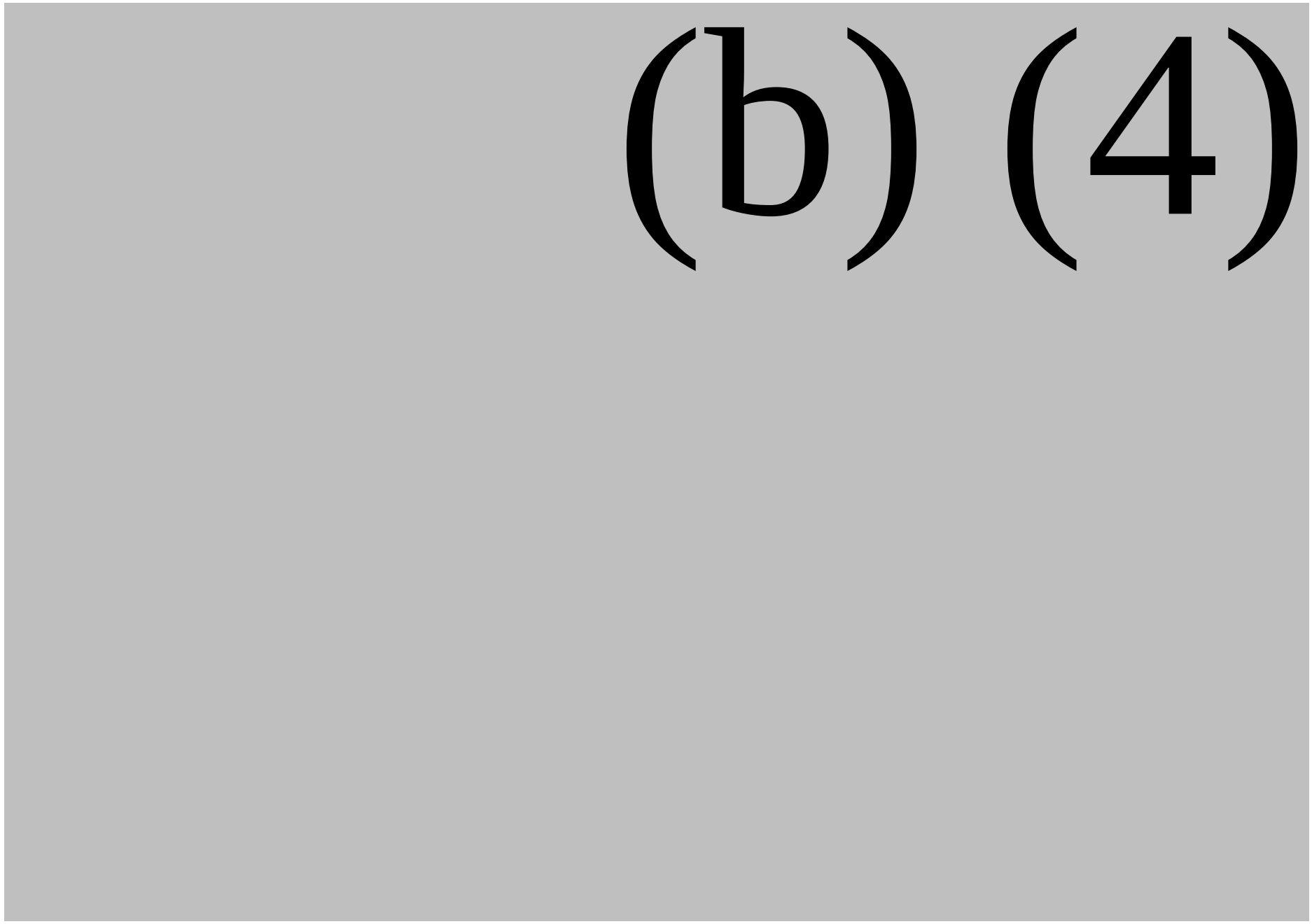
- *Specificity*
- *Accuracy*
- *Precision-repeatability*
- *Precision-intermediate precision*
- *Precision- reproducibility*
- *Limit of detection (LOD)*
- *Limit of quantification (LOQ)*
- *Linearity*

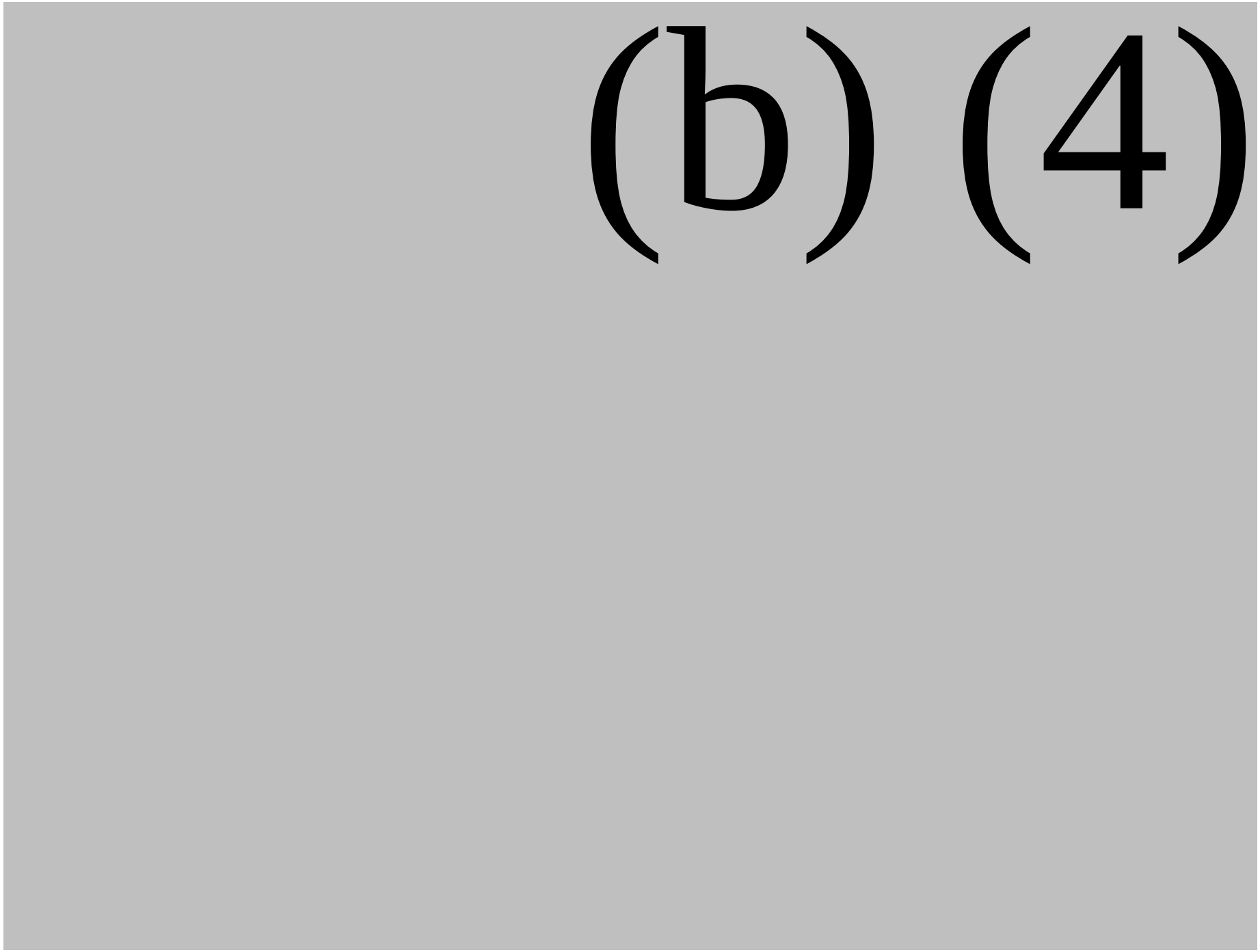
- *Range*
- *Robustness (Ruggedness)*

SOP Number	SOP Title	Parameter	Citation	(b) (4) Accredited (Y/N)	LOD	Example Chromatogram (If applicable)	Notes / Method Summary




(b) (4)


(b) (4)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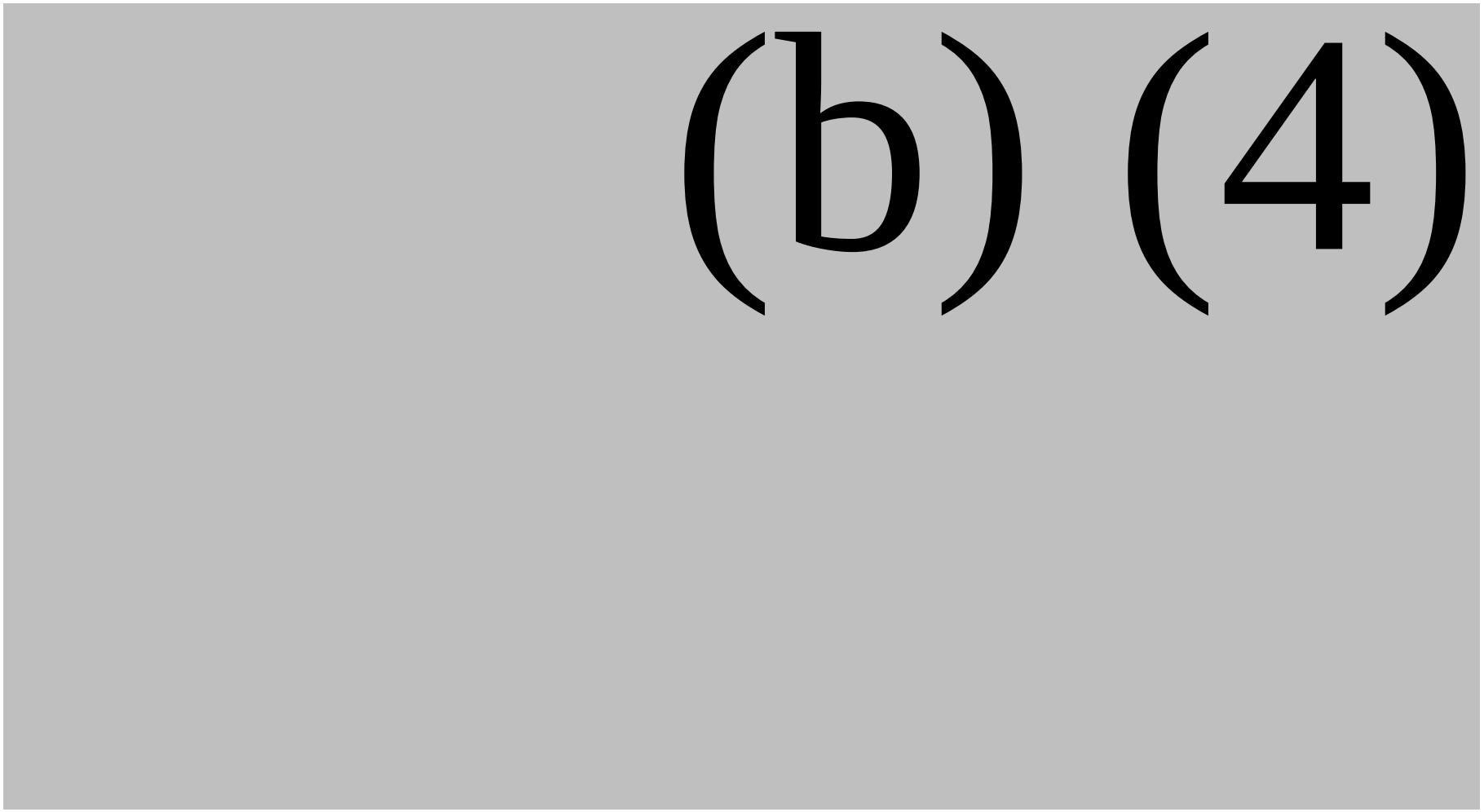

(b) (4)

Appendix 5

PT Scheme Results									
Test Date	Sample Number	PT Scheme	Round	Sample	Analyte	PT Mean	Our Result	PT SD	Z Score
1/23/2019	1853520	AAFCO	201921	Equine Feed	Tyrosine %	0.4215	(b) (4)	0.0426	(b) (4)
2/20/2019	1868925	AAFCO	201922	Porcine Feed	Tyrosine %	0.6065		0.0621	
4/12/2019	1891583	AAFCO	201923	Dry Dog Food	Tyrosine %	0.6649		0.0844	
5/3/2019	1915025	AAFCO	201924	Goat Feed	Tyrosine %	0.4261		0.0542	
5/24/2019	1939593	AAFCO	201925	Alfalfa Pellets	Tyrosine %	0.4522		0.0368	
6/14/2019	1954770	AAFCO	201926	Poultry Feed	Tyrosine %	0.6363		0.0743	
7/22/2019	1974632	AAFCO	201927	Beef Feed	Tyrosine %	0.5349		0.0569	
8/27/2019	1992161	AAFCO	201928	Catfish Feed	Tyrosine %	0.9977		0.1219	
9/24/2019	2016130	AAFCO	201929	Sheep Feed	Tyrosine %	0.3332		0.031	
10/30/2019	2037516	AAFCO	201930	Dairy Feed	Tyrosine %	0.5429		0.0554	
11/12/2019	2056173	AAFCO	201931	Llama Feed	Tyrosine %	0.5065		0.0955	
1/2/2020	2080845	AAFCO	201932	Pig Feed	Tyrosine %	0.4843		0.0574	
1/31/2020	2101307	AAFCO	202021	Chicken Layer Feed	Tyrosine %	0.6207		0.0351	
2/18/2020	2116546	AAFCO	202022	Horse Feed	Tyrosine %	0.3468		0.0345	
3/10/2020	2138057	AAFCO	202023	Dry Cat Feed	Tyrosine %	1.116		0.0926	
5/4/2020	2170267	AAFCO	202024	Cattle Feed	Tyrosine %	0.5904		0.0886	
6/15/2020	2179964	AAFCO	202025	Goat Feed	Tyrosine %	0.5115		0.0799	
7/1/2020	2196511	AAFCO	202026	Pig Grower	Tyrosine %	0.4797		0.0654	
8/5/2020	2219801	AAFCO	202027	Beet Pulp	Tyrosine %	0.3312		0.0365	
9/1/2020	2242904	AAFCO	202028	Cattle Feed	Tyrosine %	0.4156		0.0621	
9/30/2020	2262473	AAFCO	202029	Chicken Feed	Tyrosine %	0.6714		0.1184	
11/3/2020	2281644	AAFCO	202030	Sheep Feed	Tyrosine %	0.3144		0.0676	
11/20/2020	2297418	AAFCO	202031	Tortoise Feed	Tyrosine %	0.502		0.086	
11/24/2020	2308884	BIPEA - Petfood (67a)	7-1067	Crushed Corn	Tyrosine g/100g	0.31		0.03	
1/12/2021	2323477	AAFCO	202032	Swine Feed	Tyrosine %	0.4418		0.071	
1/12/2021	2346610	BIPEA - Petfood (67a)	8-1367	Wet Dog Food	Tyrosine g/100g	0.31		0.03	
1/12/2021	2346638	BIPEA - Petfood (67a)	7-1567	Fish Meal	Tyrosine g/100g	2.17		0.11	
2/11/2021	2346429	AAFCO	202121	Equine Feed	Tyrosine %	0.4091		0.05	

(b) (4)
May 2019

Shelf Life Testing of FeedKind® Interim Report

Introduction

Samples of FeedKind® have been stored under controlled conditions for 52 weeks. Samples remain under test conditions for each batch being tested and the final samples are expected to be removed from test after 156 weeks. The shelf life trial will be conducted over 156 weeks which is longer than the expected shelf life of FeedKind® and will generate sufficient data to accurately predict the shelf life of FeedKind®. This interim report will focus on the stability of the proximate components of FeedKind® crude protein, crude fat, crude fibre, ash and moisture. The final report will include details of the amino acids, fatty acids and microbiology over the full test period.

Experimental plan

Samples have been chosen at random from different batches of FeedKind® produced at the (b)(6) Market Introduction Facility (MIF) during each production run:

Reference Number	Batch	Test Conditions	Start Date
Stability Test 01	TEES004/29	25°C/60%RH*	12 October 2017
Stability Test 02	TEES004/29	40°C/75%RH*	12 October 2017
Stability Test 03	TEES004/29a	25°C/60%RH	18 October 2017
Stability Test 04	TEES004/29a	40°C/75%RH	18 October 2017
Stability Test 05	TEES004/11	25°C/60%RH	19 October 2017
Stability Test 06	TEES004/11	40°C/75%RH	19 October 2017
Stability Test 07	TEES005/28	25°C/60%RH	10 January 2018
Stability Test 08	TEES005/28	40°C/75%RH	10 January 2018

*Not heat killed.
The MIF broth is processed through a heat kill step before spray drying to kill any active bacteria this step was omitted on TEES004/29 as a trial to investigate if removing the heat kill step has an impact on the final FeedKind®.

A single bag from each batch was separated into 20 X 500g samples one sample was tested and the remaining samples placed in temperature and humidity controlled cabinets at 25°C/60%RH and 40°C/75%RH.
The sample containers used are HDPE, to replicate the PE bulk sacks that may be used at a commercial scale. Holes have been drilled in the lids to allow air into the sample container to represent leakage or absorption at full scale.

The sample plan below is being followed:

0 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.
4 Weeks	Proximate, microbiology, biogenic amines.
8 Weeks	Proximate, microbiology, biogenic Amines.

12 Weeks	Proximate, microbiology, biogenic Amines.
26 Weeks	Proximate, microbiology, biogenic Amines.
39 Weeks	Proximate, microbiology, biogenic Amines.
52 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.
78 Weeks	Proximate, microbiology, biogenic amines.
104 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.
156 weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.

NOTE: Proximate testing is for crude protein, crude fat, ash, moisture and crude fibre.

Results

The results summary below includes data for the proximate testing on all the samples under test for the first 12 months of the stability test.

Stability Test 01 TEES004/29 25°C/60%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min (b) (4)	Max 1%	Max (b) (4)
0					
4					
8					
12					
26					
39					
52					

(b) (4)

Table 1

Stability Test 02 TEES004/29 40°C/75%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min (b) (4)	Max 1%	Max (b) (4)
0					
4					
8					
12					
26					
39					
52					

(b) (4)

Table 2

Stability Test 03 TEES004/29a 25°C/60%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min (b) (4)	Max 1%	Max (b) (4)
0					
4					
8					
12					
26					
39					
52					

(b) (4)

Table 3

Stability Test 04 TEES004/29a 40°C/75%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min (b) (4)	Max 1%	Max (b) (4)
0					
4					
8					
12					
26					
39					
52					

(b) (4)

Table 4

Stability Test 05 TEES004/11 25°C/60%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min (b) (4)	Max 1%	Max (b) (4)
0					
4					
8					
12					
26					
39					
52					

(b) (4)

Table 5

Stability Test 06 TEES004/11 40°C/75%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min (b) (4)	Max 1%	Max (b) (4)
0					
4					
8					
12					
26					
39					
52					

(b) (4)

Table 6

Stability Test 07 TEES005/28 25°C/60%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min (b) (4)	Max 1%	Max (b) (4)
0					
4					
8					
12					
26					
52					

(b) (4)

Table 7

Stability Test 08 TEES005/28 40°C/75%RH

Test Duration	Moisture %	Crude Fat %	Crude Protein %	Crude Fibre %	Ash %
Weeks	Max 8%	Min 5%	Min (b) (4)	Max 1%	Max (b) (4)
0					
4					
8					
12					
26					
52					

(b) (4)

Table 8

Discussion

The only significant changes observed in the composition of the samples under test are the moisture and protein levels.

FeedKind® absorbs moisture from the atmosphere over time which can lead to an out of specification result. Table 9 below shows when the moisture and protein values fall outside specification limits.

Test	Moisture	Protein
	Weeks to Out of Specification Result	
ST01	(b) (4)	
ST02		
ST03		
ST04		
ST05		
ST06		
ST07		
ST08		

Table 9

The higher moisture content has an impact on the protein level in the sample as the FeedKind® moisture content increases during storage from say 8 to 10% the amount of moisture present in a given sample means less of the other components are present in the sample.

E.g. 100g FeedKind® with 8% moisture contains 8g of water and 92g of other components.

100g FeedKind® with 10% moisture contains 10g of water and 90g of other components.

Consequently, less of the other components are present in the sample with a higher moisture content this appears to have an impact on the protein level in the sample. The sample does not lose protein but gains moisture.

However, there is no significant change in the protein level when calculated on the dry basis.

The protein levels calculated on a dry basis are summarised in table 10.

Test	Protein (dry Basis)	
	Week 1	Week 52
ST01	(b) (4)	
ST02		
ST03		
ST04		
ST05		
ST06		
ST07		
ST08		

Table 10

The packaging material used for the FeedKind® can be selected to minimise the moisture the finished product is exposed to and help to maintain the moisture content within specification.

Conclusion

The initial findings of the shelf life study show FeedKind® to be a stable but hydroscopic product. The final product can be tested before use and any increase in moisture can be compensated for during feed production if necessary.
The data from the shelf life study will be used to confirm the shelf life of FeedKind® and determine the specification for the FeedKind®.

The final report will be issued when the shelf life tests have been completed.

(b)(6)

May 2019

Supplemental FeedKind® Shelf Life Report

Introduction

Samples of FeedKind® have been stored under controlled conditions for 52 weeks. Samples remain under test conditions for each batch being tested and the final samples are expected to be removed from test after 156 weeks. The shelf life trial will be conducted over 156 weeks which is longer than the expected shelf life of FeedKind® and will generate sufficient data to accurately predict the shelf life of FeedKind®. This supplemental report will focus on the stability of the amino acids, fatty acid, biogenic amines and microbiology of FeedKind®. The final report will be issued when the samples have completed the full test period of 156 weeks.

Experimental Plan

Samples have been chosen at random from different batches of FeedKind® produced at the Teesside UK Market Introduction Facility (MIF) during each production run:

Reference Number	**Batch**	**Test Conditions**	**Start Date**
Stability Test 01	TEES004/29	25°C/60%RH*	12 October 2017
Stability Test 02	TEES004/29	40°C/75%RH*	12 October 2017
Stability Test 03	TEES004/29a	25°C/60%RH	18 October 2017
Stability Test 04	TEES004/29a	40°C/75%RH	18 October 2017
Stability Test 05	TEES004/11	25°C/60%RH	19 October 2017
Stability Test 06	TEES004/11	40°C/75%RH	19 October 2017
Stability Test 07	TEES005/28	25°C/60%RH	10 January 2018
Stability Test 08	TEES005/28	40°C/75%RH	10 January 2018

*Not heat killed.
The MIF broth is processed through a heat kill step before spray drying to kill any active bacteria this step was omitted on TEES004/29 as a trial to investigate if removing the heat kill step has an impact on the final FeedKind®.

A single bag from each batch was separated into 20 X 500g samples one sample was tested and the remaining samples placed in temperature and humidity controlled cabinets at 25°C/60%RH and 40°C/75%RH.
The sample containers used are HDPE, to replicate the PE bulk sacks that may be used at a commercial scale. Holes have been drilled in the lids to allow air into the sample container to represent leakage or absorption at full scale.

The sample plan below is being followed:
0 Weeks Proximate, microbiology, amino acid profile, fatty acid profile,

	biogenic amines.
4 Weeks	Proximate, microbiology, biogenic amines.
8 Weeks	Proximate, microbiology, biogenic Amines.
12 Weeks	Proximate, microbiology, biogenic Amines.
26 Weeks	Proximate, microbiology, biogenic Amines.
39 Weeks	Proximate, microbiology, biogenic Amines.
52 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.
78 Weeks	Proximate, microbiology, biogenic amines.
104 Weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.
156 weeks	Proximate, microbiology, amino acid profile, fatty acid profile, biogenic amines.

NOTE: Proximate testing is for crude protein, crude fat, ash, moisture and crude fibre.

Amino acid results summary up to 52 week time point.

ST01 TEES004/29 25°C/ 60%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.7	1.7	2.9	2.2	7.3	3.4	4.6	3.6
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	2.9	5.0	1.6	2.8	1.4	3.7	4.2	2.8	1.0
52	(b) (4)								

Table 1

ST02 TEES004/29 40°C/ 75%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.7	1.7	2.9	2.2	7.3	3.4	4.6	3.6
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	2.9	5.0	1.6	2.8	1.4	3.7	4.2	2.8	1.0
52	(b) (4)								

Table 2

ST03 TEES004/29a 25°C/ 60%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.8	1.7	2.9	2.2	7.5	3.4	4.7	3.7
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	3.0	5.1	1.7	2.9	1.5	3.8	4.3	2.8	0.9
52	(b) (4)								

Table 3

ST04 TEES004/29a 40°C/ 75%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.8	1.7	2.9	2.2	7.5	3.4	4.7	3.7
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	3.0	5.1	1.7	2.9	1.5	3.8	4.3	2.8	0.9
52	(b) (4)								

Table 4

ST05 TEES004/11 25°C/ 60%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.6	1.7	2.9	2.2	7.3	3.3	4.5	3.6
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	2.9	5.0	1.5	2.8	1.4	3.6	4.1	2.8	0.9
52	(b) (4)								

Table 5

ST06 TEES004/11 40°C/ 75%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	5.6	1.7	2.9	2.2	7.3	3.3	4.5	3.6
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	2.9	5.0	1.5	2.8	1.4	3.6	4.1	2.8	0.9
52	(b) (4)								

Table 6

ST07 TEES005/28 25°C/ 60%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	6.4	1.9	3.2	2.4	7.9	3.7	5.1	4.1
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	3.3	5.5	2.0	3.1	1.6	4.2	4.5	3.0	
52	(b) (4)								

Table 7

ST08 TEES005/28 40°C/ 75%RH

Weeks	Cystine %	Aspartic %	Methionine %	Threonine %	Serine %	Glutamic %	Glycine %	Alanine %	Valine %
0	0.4	6.4	1.9	3.2	2.4	7.9	3.7	5.1	4.1
52	(b) (4)								

Weeks	Iso-Leucine %	Leucine %	Tyrosine %	Phenylalanine %	Histidine %	Lysine %	Arginine %	Proline %	Tryptophan %
0	3.3	5.5	2.0	3.1	1.6	4.2	4.5	3.0	
52	(b) (4)								

Table 8

Amino Acid Discussion

The amino acid profile of the samples being tested under both storage conditions showed no significant changes in the first 52 weeks of the shelf life study.

The amino acid profile will be tested again at the 104 & 156 week time points.

Fatty acid profile results summary up to 52 week time point.

ST01 TEES004/29 25°C/ 60%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.79	3.50	2.17	0.07
52				(b) (4)

Table 9

ST02 TEES004/29 40°C/ 75%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.58	3.53	2.42	0.00
52				(b) (4)

Table 10

ST03 TEES004/29a 25°C/ 60%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.91	3.40	2.38	0.03
52				(b) (4)

Table 11

ST04 TEES004/29a 40°C/ 75%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.91	3.40	2.38	0.03
52				(b) (4)

Table 12

ST05 TEES004/11 25°C/ 60%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	2.14	3.36	2.28	0.03
52				(b) (4)

Table 13

ST06 TEES004/11 40°C/ 75%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	2.14	3.36	2.28	0.03
52				(b) (4)

Table 14

ST07 TEES005/28 25°C/ 60%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.58	3.15	2.36	0.02
52				(b) (4)

Table 15

ST08 TEES005/28 40°C/ 75%RH

Weeks	Unidentified %	Saturated %	Monounsaturated %	Polyunsaturated %
0	1.58	3.15	2.36	0.02
52				(b) (4)

Table 16

Fatty Acid Discussion

The fatty acids profile showed no significant changes over the first 52 weeks of the shelf life test. The fatty acid profile will be tested again at the 104 & 156 weeks time points.

Biogenic amine results summary up to 52 Week time point.

ST01 TEES004/29 25°C/ 60%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	231	5150	<5	<5
4						
8						
12						
26						
39						
52						

(b) (4)

Table 17

ST02 TEES004/29 40°C/ 75%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	231	5150	<5	<5
4						
8						
12						
26						
39						
52						

(b) (4)

Table 18

ST03 TEES004/29a 25°C/ 60%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	7	4599	<5	<5
4	(b) (4)					
8						
12						
26						
39						
52						

Table 19

ST04 TEES004/29a 40°C/ 75%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	7	4599	<5	<5
4	(b) (4)					
8						
12						
26						
39						
52						

Table 20

ST05 TEES004/11 25°C/ 60%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	<5	4471	<5	<5
4	(b) (4)					
8						
12						
26						
39						
52						

Table 21

ST06 TEES004/11 40°C/ 75%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	<5	4471	<5	<5
4	(b) (4)					
8						
12						
26						
39						
52						

Table 22

ST07 TEES005/28 25°C/ 60%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	<5	4953	<5	<5
4	(b) (4)					
8						
12						
26						
52						

Table 23

ST08 TEES005/28 40°C/ 75%RH

Duration	Putrescine	Histamine	Cadaverine	Spermidine	Tyramine	Spermine
Weeks	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
0	<5	<5	<5	4953	<5	<5
4	(b) (4)					
8						
12						
26						
52						

Table 24

Biogenic Amine Discussion

The putrescine concentration starts to increase after 26 weeks on test however the spermidine concentration decreases over time. The other biogenic amines histamin, cadaverine, tyramine and spermine remained below detection limits.

Cadaverine is present in ST01 and ST02 which is the material which was not heat killed during production the concentration of cadaverine will be monitored for the duration of the shelf life study.

The biogenic amines will be tested again at the 78, 104 & 156 weeks time points.

Microbiology results summary up to 52 week time point.

Stability Test 01 TEES004/29 25°C/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	6100	170000	<10	40
4	(b) (4)			
8				
12				
26				
39				
52				

Table 25

Stability Test 02 TEES004/29 40°C/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	6100	170000	<10	40
4	(b) (4)			
8				
12				
26				
39				
52				

Table 26

Stability Test 03 TEES004/29a 25°C/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	280	350	<10	10
4	(b) (4)			
8				
12				
26				
39				
52				

Table 27

Stability Test 04 TEES004/29a 40°C/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	280	350	<10	10
4	(b) (4)			
8				
12				
26				
39				
52				

Table 28

Stability Test 05 TEES004/11 25°C/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	170	240	<10	<10
4	(b) (4)			
8				
12				
26				
39				
52				

Table 29

Stability Test 06 TEES004/11 40°C/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	170	240	<10	<10
4	(b) (4)			
8				
12				
26				
39				
52				

Table 30

Stability Test 07 TEES005/28 25°C/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	10	20	70	<10
4	(b) (4)			
8				
12				
26				
52				

Table 31

Stability Test 08 TEES005/28 40°C/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	10	20	70	<10
4	(b) (4)			
8				
12				
26				
52				

Table 32

Discussion

Overall there is no significant change in the microbiology of the samples under test. There is an increase in total viable count (TVC) at weeks 26 and 52 for Stability tests 07 and 08. Nevertheless, more time points are required to determine if this is a significant trend. A high number of yeasts was observed at week 12 in Stability test 08. However, compared to the results from other time points in this test, it appears spurious in nature.
The microbiological activity in the samples will be tested again at the 78, 104 & 156 weeks time points.

Conclusion

The initial findings of the shelf life study show FeedKind® to have a stable amino acid and fatty acid profile. No significant change has been observed in the biogenic amines and microbiological components of the samples under test.

The final report will be issued when the shelf life tests have been completed.

Shelf Life Testing of FeedKind® Interim Micro Report

Introduction

Samples of FeedKind® have been stored under controlled conditions for 52 weeks. Samples remain under test conditions for each batch being tested and the final samples are expected to be removed from test after 156 weeks. The shelf life trial will be conducted over 156 weeks which is longer than the expected shelf life of FeedKind® and will generate sufficient data to accurately predict the shelf life of FeedKind®.

This interim report covers the microbiology results of the study to date.

Results

Stability Test 01 TEES004/29 25ºC/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	6100	170000	<10	40
4	(b) (4)			
8				
12				
26				
39				
52				
78				

Stability Test 02 TEES004/29 40ºC/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	6100	170000	<10	40
4	(b) (4)			
8				
12				
26				
39				
52				

Stability Test 03 TEES004/29a 25°C/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	280	350	<10	10
4				
8				
12				
26				
39				
52				

(b) (4)

Stability Test 04 TEES004/29a 40°C/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	280	350	<10	10
4				
8				
12				
26				
39				
52				

(b) (4)

Stability Test 05 TEES004/11 25°C/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	170	240	<10	<10
4				
8				
12				
26				
39				

(b) (4)

52	(b) (4)

Stability Test 06 TEES004/11 40°C/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	170	240	<10	<10
4	(b) (4)			
8				
12				
26				
39				
52				

Stability Test 07 TEES005/28 25°C/60%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	10	20	70	<10
4	(b) (4)			
8				
12				
26				
52				

Stability Test 08 TEES005/28 40°C/75%RH

Test Duration (Weeks)	TVC (Anaerobic @ 30ºC) cfu/g	TVC (Aerobic @ 30ºC) cfu/g	Yeasts cfu/g	Moulds cfu/g
0	10	20	70	<10
4	(b) (4)			
8				
12				

26	(b) (4)
52	

Discussion

Overall there is no significant change in the microbiology of the samples under test. There is an increase in total viable count (TVC) at weeks 26 and 52 for Stability tests 07 and 08. Nevertheless, more time points are required to determine if this is a significant trend. A high number of yeasts was observed at week 12 in Stability test 08. However, compared to the results from other time points in this test, it appears spurious in nature.

(b) (4)

Method Summary

SOP Number : S2106

SOP Title : **Direct Enumeration of Yeasts and Moulds by the Colony Count Method using** (b) (4)

Introduction

The method described is applicable to the enumeration of yeasts and moulds in all food types, products destined for animal feed, environmental samples, swabs, composts, sludges, slurries and any other unspecified sample, using a surface colony count technique with incubation at 25°C.

Principle

The enumeration of yeasts and moulds by this method involves inoculation of the surface of the selective agar medium with a specified volume of a $10^{-1}$and other appropriate decimal dilutions of the test sample and incubation at 25°C for 5 days. Characteristic colonies are counted and the result is calculated as the colony count per gram or swab of yeasts and moulds.

(b) (4)

Storage stability of BioProtein

V.4

(b) (4)

STORAGE STABILITY OF BIOPROTEIN

Summary

The storage stability of BioProtein containing about 6 percent of moisture have been monitored at 22 and 37 °C. The samples were stored in polyethylene bags the moisture content during the storage period of 64 weeks increased at 22 °C and decreased at 37 °C due to moisture diffusion. The content of fat, protein and the essential amino acids remained fairly constant during 64 weeks of storage. Free fatty acids increased from 13 to 19 % of total fat during the first 16 weeks and then remained constant for the last 48 weeks.

Samples and storage conditions

A 10 kg sample of bioprotein, label 93010635, was received from (b) (4)

The sample was divided into nine subsamples on January 6th 1993. One of the subsamples was analysed immediately and the others were packed in airtight-closed polyethylene bags and stored for later analysis. The bags were placed in thermostated incubators - four bags at 21-23 °C and the other four at 36-38 °C. One bag was removed from each incubator and analysed after storage for 4, 16, 32 and 64 weeks.

The bags was made from 0.07 mm polyethylene, which is permeable for oxygen and carbon dioxide, but is a fairly effective barrier for water vapour.

Analytical programme

The analytical programme comprised the nutrients: Protein, essential amino acids and fat, together with the deterioration products from fat: Free fatty acids and peroxides. The approved EEC-methods were used wherever possible as stated below:

- Moisture	EF(71/393/EØF)
- Crude protein	EF(72/199/EØF)
- Crude fat (acid hydrolysis)	EF(84/4/EØF)
- Cystine, methionine, threonine and lysine	Landbr.min.met. 1.2.1
- Free fatty acids (FFA)	BI-medd. 2, 1980
- Peroxide value	BI-medd. 2, 1980

(b) (4)

Results and discussion

The analytical results on BioProtein after storage at 22 and 37 °C is shown in Table 1 and graphed in figure 1. The last column in table 1 shows the analytical precision of the results measured as the standard deviation of repeated analyses of a reference sample during a long period of time. The precision of peroxide value is just estimated by experience, because it is impossible to keep a constant value in a sample for a long time.

The variance of the present results were compared to the analytical precision by a chi-squared test at a significance level of 0.05. Only moisture and free fatty acids showed a significant variation during the storage period.

The significant changes in moisture content show that the polyethylene bags were not entirely impermeable to water vapour. The samples absorbed 1 % moisture from the surroundings at 22 °C and lost 2 % moisture at 37 °C during 64 weeks. In order to obtain comparable values, all the other parameters are calculated on dry matter basis or as percentage of protein or fat.

Crude protein and the amino acids, cystin, methionine, threonine and lysine, remained fairly constant during the storage period of 64 weeks, irrespective of the storage temperature. This shows an excellent storage stability of the protein and essential amino acids, which constitutes the main part of the feed value of BioProtein.

The content of crude fat was determined by acid hydrolysis. The rather low value after 4 weeks of storage at 22 °C deviates significantly from the other values but the deviation is not significant compared with the analytical precision. If this low value is ignored, a slight decrease of fat content during the storage period is evident from figure 1 and it might be explained by a slow deterioration of the lipids in BioProtein.

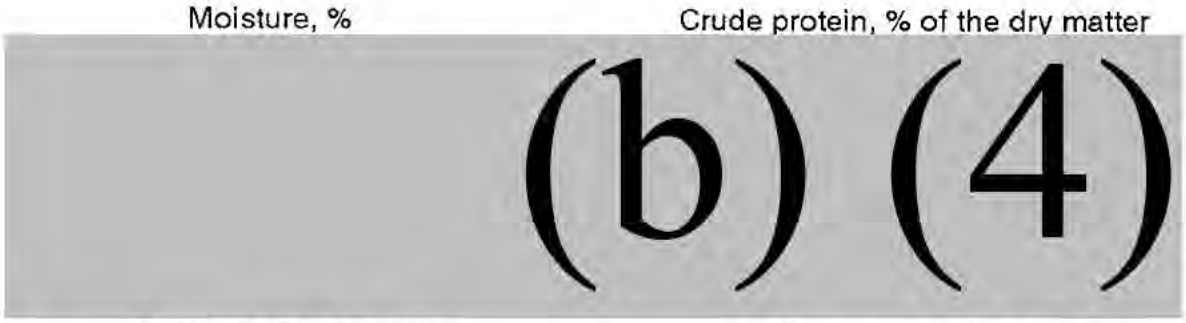
Free fatty acids is determined by titration and calculated as oleic acid, but might comprise other acids produced by hydrolysis or oxidation of lipids. The results show significantly increasing values during the first 16 weeks of storage and then constant values for the next 48 weeks. The peroxide value is very low and remains constant or slightly decreasing during storage. This shows a satisfactory oxidative stability of the fat in BioProtein and the increased level of free fatty acids is probably not of any importance for the feed value.

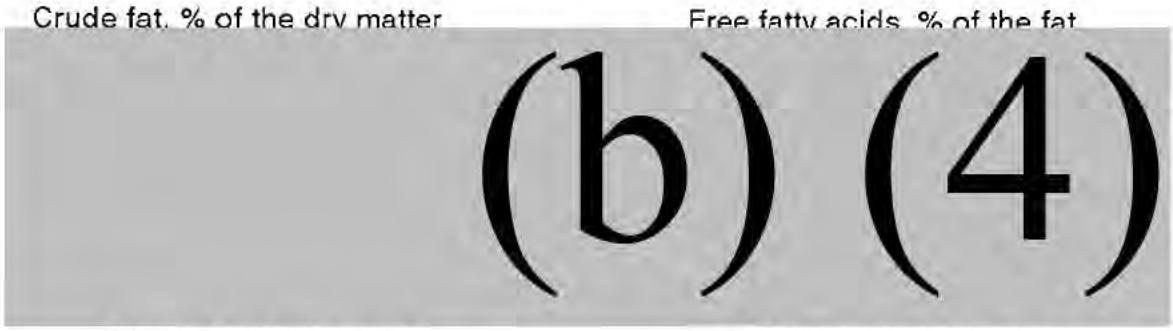
TABLE 1. Storage stability of BioProtein at 22 and 37 °C - Analytical results.

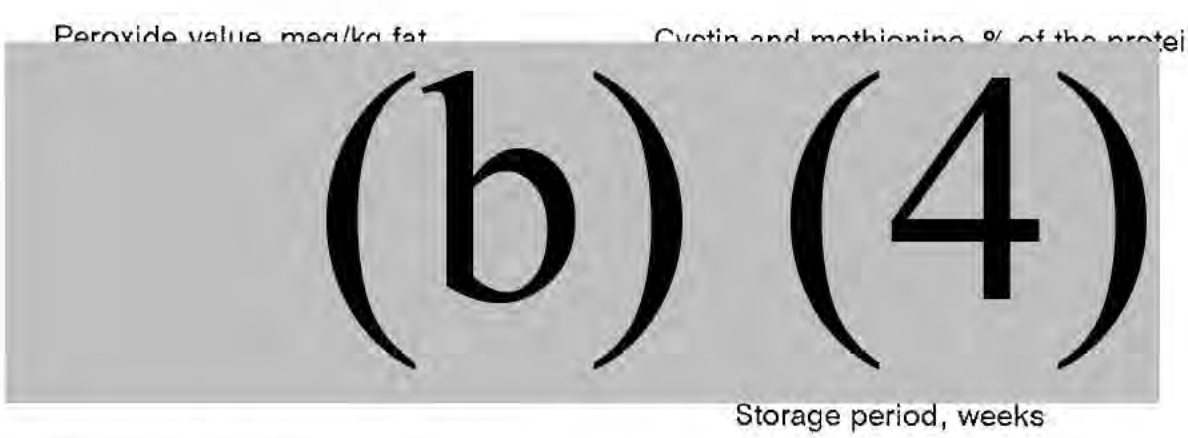
Storage temperature, °C:		22				37				Precision of analysis
Storage time, weeks:	0	4	16	32	64	4	16	32	64	SEM
Moisture, %	6.2									(b) (4)
Crude protein, % in DM	72.1									
Crude fat, % in DM	10.3									
Free fatty acids, % of fat	12.8									
Peroxide value, meq/kg fat	2.3									
Cystin, g/16 g N	0.54									
Methionine, g/16 g N	2.60									
Threonine, g/16 g N	4.45									
Lysine, g/16 g N	6.04									

(b) (4)

(b) (4)







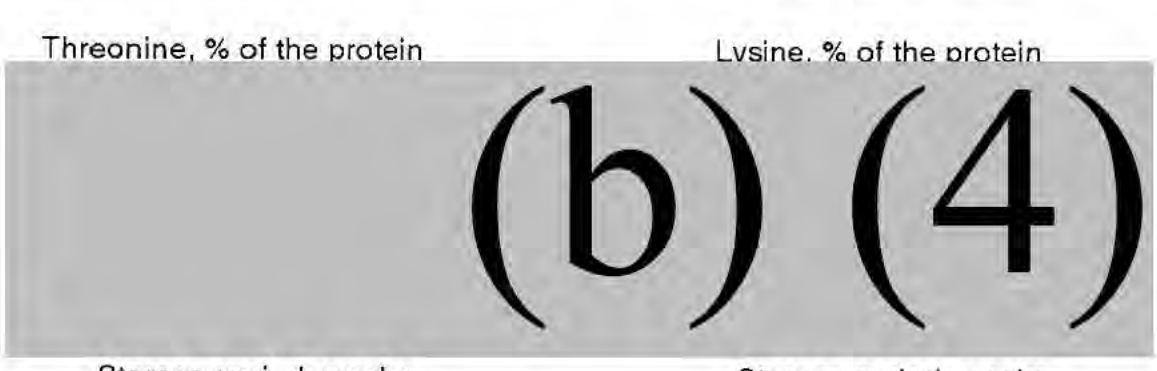


Figure 1. Quality of Bioprotein during storage at 22 C (solid line) and at 37 C (dotted line).

First report of the Scientific Committee for Animal Nutrition on Question 69 by the Commission on the use of protein products of fermentation from natural gas obtained by culture of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans, Bacillus brevis, Bacillus firmus*, the living cells of which have been killed
(Opinion expressed on 28 April 1995)

TERMS OF REFERENCE (May 1994):

The Scientific Committee for Animal Nutrition (SCAN) and the Scientific Committee for Food (SCF) are requested to give their opinion on the following questions:

1. Does the protein product of fermentation from natural gas obtained by culture of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans, Bacillus brevis, Bacillus firmus,* the living cells of which have been killed, have a nutritional value for the animal because it provides nitrogen or protein?

2. Can the use in animal nutrition of the protein product of fermentation from natural gas obtained by culture of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans, Bacillus brevis, Bacillus firmus,* the living cells of which have been killed, result in risks for humans (consumer or user) or the animal health, or be prejudicial to the environment?

3. Does the use of the protein product of fermentation from natural gas obtained by culture of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans, Bacillus brevis, Bacillus firmus,* the living cells of which have been killed, harm the consumer by impearling the distinctive features of animal products?

4. Can the above-mentioned protein product be monitored in feedingstuffs?

BACKGROUND

The Council of the European Union, when adopting Directive 82/471/EEC[1] considered it essential, before including a new product in one of the groups listed in the annex of this directive, to establish that it has the required nutritional value and that, when used sensibly, it has no detrimental effect on human or animal health or on the environment; and does not harm the consumer by impairing the distinctive features of animal products. With a view to providing all necessary guarantees, the Community procedure adopted should in certain cases of amendment of the annex make provisions for the compulsory consultation of the Committees created by Commission Decisions 74/234/CEE[2] and 76/791/EEC[3].

In accordance with the provisions laid down in the Article 6, amendments to be made to the annex as a result of developments in scientific or technical knowledge shall be adopted by the Standing Committee of Animal Nutrition in accordance with the procedure laid down in Article 13.

According to this article the Commission representative shall submit to the Standing Committee a draft of the measures to be adopted, and the Standing Committee shall deliver

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1 Concerning certain products used in animal nutrition (O.J. No. L213, 21.07.82, p. 8)
2 Instituting a Scientific Committee for Food (SCF). (O.J. No. L136, 20.05.74, p.1)
3 Instituting the Scientific Committee for Animal Nutrition (SCAN). (O.J. No. L279, 09.10.76, p.35)

their opinion on the draft within a time limit set by the chairman according to the urgency of the matter, and shall decide by majority votes. In order to ensure that the product concerned complies with the principles set out in Directive 82/471/EEC[1], a dossier prepared in accordance with the provisions of Council Directive 83/228/EEC[4] should be prepared and, if requested, be the subject of consultation of members of the above-mentioned Scientific Committees set up by the Commission. This consultation is made compulsory for bacteria and yeasts by Article which 6 establishes that in the case of the products referred to in sections 1.1 (bacteria) and 1.2 (yeasts) of the annex, the Commission shall consult the SCF and the SCAN.

A request has been made to register the protein product of fermentation from natural gas obtained by culture of *Methylococcus capsulatus* (Bath), *Alcaligenes acidovorans, Bacillus brevis, Bacillus firmus,* the living cells of which have been killed according to the conditions set out in the attached table. It should be noted that, a product of similar nature was examined previously by the SCAN, and the opinion of the Committee expressed on 23 September 1985[5] in a report established jointly by the Scientific Committee for Animal Nutrition and the Scientific Committee for Food on the use in animal nutrition of protein products obtained from bacteria of the *Methylococcaceae* family[6].

A first report on the submission for registration of the protein product of fermentation from natural gas was considered both by the SCAN and the SCF in May 1994. In this report the documentation submitted in March 1993 was reviewed and certain additional information was requested by both committees at that time before a final answer to question No. 69 could be given to the Commission. A first supplementary dossier was provided in November 1994 which answered some of the questions of the committees. At the same time the submission was changed by restricting the use of the protein product in the feed of young growing animals, e.g. piglets, calves and fish in the early stages of growth. No application for the use in chicken was made, although data for this species were included in the original submission. Even this first supplementary dossier left some of the questions previously put by the SCAN and the SCF inadequately answered. The points still at issue were again transmitted to the submitter and a further supplementary dossier was submitted in February 1995. All additional information now supplied is included in this final report.

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4 On the fixing of guidelines for the assessment of certain products used in animal nutrition. (O.J. No. L126, 13.05.83, p. 13)

5 Fifth Series 1986; Report EUR 1041EN. Catalogue N° CD-NK-86-003-EN-C. p.51

6 In particular from *Methylophilus* cultivated in methanol (Pruteen)

Proposal for inclusion into the annexes of Directive 82/471

1	2	3	4	5	6	7
Name of product group	Name of Product	Designation of nutritive principle or identity of the micro-organism	Nutrient substrate (specifications if any)	Composition characteristics of the product	Animal species	Special provisions
1.1. Bacteria 1.1.2Bacteria cultivated on natural gas	1.1.2.1 Protein product of fermentation from natural gas obtained by culture of: *Methylococcus capsulatus* (Bath) *Alcaligenes acidovorans*, *Bacillus brevis*, *Bacillus firmus*, - the cells of which have been killed	*Methylococcus capsulatus (Bath)* NCIMB strain 11132, *Alcaligenes acidovorans* NCIMB strain 12387, *Bacillus brevis* NCIMB strain 13288, *Bacillus firmus* NCIMB strain 13280	Natural gas, (91% methane, 5,1% ethane 1,9% propane 0,4% isobutane 0,5% n-butane 1,1% other minor components), ammonia, mineral salts	Nitrogen expressed as crude protein minimum 65%	- Pigs - Calves - Fish	Declaration to be made on the label or packaging of the product - the name: "Protein product obtained by fermentation of natural gas" - nitrogen expressed as crude protein - crude ash - crude fat - moisture content - instructions for use - declaration of the words "avoic inhalation" - For each animal species: recommended and maximum inclusion level expressed as percentag of the total nitrogen content of th complete feedingstuffs Declaration to be made on the label or packaging of the com-

						pound feedingstuffs: -the name: "Protein product obtained by fermentation of natural gas" - amount of the product contained in the feedingstuff"

OPINION OF THE COMMITTEE

The protein product presently being considered is another example of an edible bioprotein produced by cultivation of bacteria of the Methylococcaceae family together with certain other bacteria, for which product data on performance in livestock feeding have become available to the Committees and for which appropriate dossiers were supplied. BioProtein* is obtained by growing *Methylococcus capsulatus* (Bath), strain NCIMB-11123, aerobically on natural gas composed of 91% methane, 5.1% ethane, 1.9% propane, 0.4% isobutane, 0.5% n-butane and 1.1% other minor components. The bacteria oxidise methane through intermediate steps ending with CO_2 and thereby form a biomass. Ammonia is used as nitrogen source and appropriate mineral salts are added to satisfy the culture requirements.

The oxidation products arising during the fermentation process can inhibit, however, the growth of *Methylococcus capsulatus*, and it is therefore necessary to include other bacteria in the production line, which are able to utilise these inhibitory oxidation products. These are *Alcaligenes acidovorans DB3* (strain NCIMB 12387), *Bacillus brevis* DB4 (strain NCIMB 13288) and *Bacillus firmus DB5* (strain NCIMB 13280). These 4 bacterial strains form an ecosystem which in part ensures a stable production performance and in part protects the fermenting culture against unwanted contamination with other bacteria by occupying all niches which might be available to intruding micro-organisms.

Nutritional value

The protein product is marketed as a free-flowing non-dusty agglomerate (particle diameter 100-300 ìm). The crude protein content (Nx6.25) of the product is approximately 70% on a dry weight basis and includes minor contributions of nitrogen compounds other than protein, notable nucleic acids. The true protein content is 66% of dry weight. It also contains 9% crude fat, 7% crude ash, 7% nucleic acid, and a maximum of 100mg/kg copper. The N-free fraction is composed of 4.5% glucose, 2.4% starch and variable amounts of unidentified cellwall polysaccharides, and about 7% crude ash. The amino acid pattern, typical of a single cell protein, consists of lysine 6.5, methionine 2.8, methionine-cysteine 3.4, threonine 4.7. tryptophan 2.2, arginine 6.2, tyrosine 3.4 g/&§g N and a number of others in smaller amounts.

The true digestibility of the protein is 78.5% and the biological value 84% as determined in the rat. The NPU (net protein utilisation) is about 66% of the biological value with PU (protein utilisation = NPU x protein content) being 44.7%.

Further information on the amino acid profile of the protein product and their digestibility has now been supplied. the protein product is nutritionally characterised by a high protein content and a well balanced amino acid profile compared with other protein sources used in animal feeds.

The content of essential and semi-essential amino acids has been compared with that of fish meal, meat and bone meal, meat meal, blood meal, soybean meal, soyprotein concentrate and rape seed meal. The levels of methionine and cystine, the S-containing amino acids, and the lysine and arginine are somewhat lower, while the levels of threonine, tryptophan, leucine, isoleucine and phenylalanine are higher than those found in fishmeal (Pedersen et al., 1994).

The digestibility of the amino acids has also been studied in mink, pigs and poultry (Skrede *et al.*, 1994) and values for veal calves and salmon are supplied in the second supplementary dossier (February, 1995). The D-amino acids are most likely to derive from the peptidoglycan murein that is present in the bacterial wall. They do not present a nutritional problem for the target species.

Information has now been supplied on the composition of the lipid fraction of the protein product. New data were obtained by using gas chromatography combined with mass spectrometry and a full list of all fatty acids present including cyclopropanoic acid (cycC17:0 or 9,10 methylene hexadecanoic acid) is now available. The corrected cyclopropanoic acid content is now calculated to be about 1.6%. In a three months feeding study in pigs no accumulation of cyclopropanoic acids was demonstrable in the body fat. The content of n-3 fatty acids in fish fed the protein product shows only a small reduction in C 22:5 and C 22:6 fatty acids when the diet included 37% of the protein product, allowing the conclusion that the cyclopropanoic acid does not interfere significantly with the elongases and desaturases involved in the synthesis of the nutritionally important n-3 fatty acids in salmon fed the protein product.

Apart from the sensory evaluation of chicken meat which was supplied in the original submission sensory evaluation data have now been made available for salmon fed for 2 years four diets containing the protein product and also for the back fat of pigs fed the protein product for 100 days. In neither instance was a negative effect on the quality of the edible products from animals and fish fed the protein product noted.

The protein product has been tested for its efficacy and nutritive value only in trials conducted under laboratory conditions. No field trials with pigs and veal calves are available, and no trials at all with calves starting at birth weight The adverse effect of 12% inclusion for veal calves is probably the result of nutritional imbalance. The protein product is proposed to be used at 8% in the feed of calves as a replacement of some of the skimmed milk in milk feeds. An experiment was carried out over 10 days to study the acceptability and functionality of calf milk replacers containing 10%, 20% and 30% protein product.

The results showed no acceptability problems arising even with a dietary incorporation of 30% bioprotein. But these experiments were not carried out with calves at birth weight. The protein product was, however, found to be unstable in calf milk replacers and caused faeces to be stiffer and to have an aberrant grey green colour.

In a second experiment on the digestibility of including either 10% or 20% of the protein product in calf feed there was a significant reduction in apparent digestibility of the crude protein (72% against 92%) and a similar reduction in the digestibility of carbohydrates (38% against 98%) but no effect on the digestibility of fat (95%) compared to the control diet. Only at the 10% inclusion level were daily weight gains and feed conversion ratios comparable to the control diet. The digestibilities of the amino acids were well below (10%-20%) those of skim milk.

In a third combined balance and growth study in calves 4%, 8%, 12%, 18% and 24% of the protein product were used in combination with 44%, 38%, 32%, 26% and 14.6% skimmed milk powder.

The feeds were also corrected for lysine, methionine and threonine content. Inclusion levels above 12% reduced the digestibility of the test diets. Weight gain, nitrogen retention, metabolisable energy content (2.8 Mcal/kg bioprotein against 4.5 Mcal/kg casein) and feed conversion ratio were reduced.

The nutritive value of the protein product for pigs in terms of protein and energy value was studied in piglets and compared with that of fishmeal. In a second experiment the protein digestibility of the bioprotein was 72% and the net energy was 8.96 MJ/kg.

In a 28 day study, using 120 weaned piglets aged 28 days, either 4%, 8% or 12% of the protein product were included in the feed. Daily weight gain was highest when 4% bioprotein were included (1 kg bioprotein replaced 0.85 kg LT fishmeal + 0.15 kg cereals, extra lysine was added to meet Danish recommendations). Inclusion of 12% yielded a reduced feed conversion ratio. These results support the inclusion of 8% protein product in pig feed as replacement for fishmeal, a level causing no adverse effects on the performance and health of piglets. However no dose-response studies over longer periods are available and no large field trials exist with 8% inclusion levels in pig feeds in EU countries.

The nutritive value of bioprotein for Atlantic salmon was studied in several trials. Fish weighing 70 g were maintained in seawater and fed feed with inclusion levels of 6.5%, 13% and 26% of the protein product. Growth was good and showed no difference in daily weight gains and feed conversion ratio between the groups. The highest inclusion level produced a tendency for reduced growth but no explanation is given for this effect.

In another dose-response trial in fresh water using fish of average weight of 650 g, up to 36% replacement of fish protein by the protein product caused no significant depression in growth rate compared to controls. Feed conversion ratio was also unaffected and no pathological effects attributable to the bioprotein were noted. At higher inclusion levels weight gain and feed conversion ratio were impaired.

Significant differences in growth were observed with atlantic salmon, in sea water with 19,3% of the protein product inclusion against 36% inclusion in the diet for 14 months from first feeding. Optimal inclusion levels averages 20%. This means that 25% of dietary amino acids can be provided by the protein product.

In a trial on juvenile fish weighing 60 g the protein and amino acid digestibility decreased linearly with increasing inclusion levels of 5%, 9.9%, 19.3% and 37% of the protein product. Fat digestibility and metabolisable energy of the diet also decreased but starch digestibility increased. The palatability of the composite feed was as good as that of the conventional fishmeal diet.

In a further diet performance trial over 4 periods, each of 28 days, in juvenile salmon weighing 0.2 g similar growth was obtained with up to 20% protein replacement by the protein product. Inclusion of 37% reduced growth and caused higher though unexplained mortality during the starting feeding period. The digestibility of the protein product was calculated to be 79%, of the fat 78% and of the starch 68%. There is insufficient information about the metabolism of the non-protein nitrogen fraction to estimate the metabolisable energy or net energy.

In another test using salmon weighing between 50 and 64 g there was no difference from controls with regard to growth and digestibility of the protein product for the first month. After 3 months the group fed the protein product showed better growth and better digestibility of amino acids. No adverse effects were seen with inclusion of 19% after 6 months. Fin erosion was noted in all groups.

The inclusion level of 19-20% in salmon diets (this means an inclusion of 14% protein) from Bioprotein seems the optimum. Seawater fishes support 35% inclusion level without differences in growth rate and feed conversion rate.

On the basis of the foregoing information the Committees concluded that the protein product appears to have a good nutritional value and to be an adequate protein source for fish. In pigs and calves adverse results occurred when the inclusion in feeds exceeds certain values.

The role of the comparatively high nucleic acid content and the high ash content is unclear and with some species, such as chicken, a palatability problem is apparent. No quantitative information has been supplied on the presence of D-amino acids but their presence is not regarded as of nutritional importance. The available efficacy trials are few in number, are only carried out in experimental groups, and are of short duration. No field trials have been reported. There are no efficacy trials in calves at birth weight nor in piglets weaned before 28 days of age.

Evaluation of risks

Methylococcus capsulatus (Bath), the bacterial strain used for the production of the protein product, is the type species for the family of Methylococcaceae. It represents about 90% of the stock culture. It occurs naturally in aerobic environments where methane is available. Growth occurs at temperatures between 37°C and 52°C. It is not known to be pathogenic or toxicogenic. Because it grows only on methane and at high temperatures pathogenic effects cannot be expected.

Alcaligenes acidovorans DB3 belongs to the family of *Pseudomonadaceae* and represents about 8% of the stock culture. The description *Comamonas acidovorans DB3* does not appear in Bergey's Manual (1984). This bacterium also occurs naturally and it is not known to carry virulence plasmids. The non-pathogenicity of this bacterium is further supported by the results of experiments carried out on mice by i.v. injection of 10^4 - 10^9 viable cells/kg b.w. Weight gain was not influenced by this treatment. No treatment-related macroscopic pathological changes were observed.

Bacillus brevis DB4 belongs to the genus Bacillus, but the species used is heterogeneous. It represents about 1% of the stock culture. The strain used in the manufacture of the protein product also occurs naturally and does not carry any known virulence plasmids. The non-pathogenicity is supported by the results of experiments carried out on mice by t.v. injection of 10^4 - 10^9 viable cells/kg b.w. There were no adverse effects on weight gain. One mouse died with signs of shock following the i.v. injection. No treatment-related macroscopic pathological changes were observed. However, food poisoning has been performed with a suspension of washed bacteria and oral test for toxin production is needed.

Bacillus firmus DB5 belongs to the genus Bacillus and the species used is heterogeneous. The strain occurs naturally and does not carry any known virulence plasmids. It represents about 1% of the stock culture. the non-pathogenicity is supported by the results of experiments on mice using i.v. injection of 10^4 - 10^9 viable cells/kg b.w. There were no adverse effects on weight gain and no mortalities. No treatment-related macroscopic pathological changes were observed.

The protein product stock and production cultures are kept freeze-dried and are regularly tested for composition and for the presence of pathogenic contaminants. Pathogenic risks for man or animals can only be expected, if viable cells of the production strains escape the fermenter or the sterilization procedure of the final product is inadequate. Because the optimum temperatures of the bacterial strains used all exceed 37°C and of the need for special growth requirements as well as the experimental evidence of non-pathogenicity it is unlikely that these bacteria can act as human or animal pathogens.
Heat-stable toxic substances are not expected in the sterilised product because of the absence of adverse effects in the feeding trials with calves, piglets, chicken and salmon.

The protein product is a sterile product as concerns the bacteria used for production of the biomass. The commercial product is highly contaminated but the type and number of contaminating micro-organisms do not differ from those found in similar bioproteins.

All production strains are genetically stable and unlikely to mutate into antibiotic-producing or toxin-producing strains. The fermentation product is regularly checked for the presence of toxins and antibiotics. The protein product does not constitute a microbiological risk to animals or man. Accidental release into the environment will have no deleterious consequences as all organisms of the production culture are already present in natural habitats. Nevertheless, the *Bacillus brevis* strain used should be tested orally to exclude the possibility of toxin production.

No immunological monitoring of workers exposed to the protein product has been carried out. No data have been supplied on possible exposure to dust from the spray-dried biomass or the agglomerated product.

Effects in target species

A feeding study in calves, starting at weight 110 kg, extending over 7 weeks, with 4%-24% protein product in the diet produced no significant toxicological effects, although inclusion levels at and above 18% reduced weight gain and feed conversion ratio and caused difficulties with digestion. The latter showed itself as faeces of reduced stiff consistency and a reduced feed intake. Apparent faecal digestibility coefficients of dry matter, ash, organic matter, crude protein, crude fat, carbohydrates, and iron decreased with increasing inclusion percentage. Nitrogen utilisation decreased similarly. Utilisation of ingested iron increased with increasing inclusion of the bioprotein. No clinical parameters were reported. The NEL appeared to be the inclusion level of 12% in the diet.

A feeding study in piglets, aged 28 days and weighing 25 kg, extending over 4 weeks, with 4%-12% protein product in the diet showed no significant effects on health, appetite and daily weight gain. At the 12% inclusion level the feed conversion ratio was significantly smaller. No

clinical parameters were reported. The NEL appeared to be a replacement level of 8% fishmeal in the diet.

Some 5 feeding studies in salmon, extending over 14-20 weeks, with 5%-70% protein product in the diet showed good growth and no effect on the feed conversion ratio up to the 33% inclusion level. No signs of hepatotoxicity attributable to the protein product were noted. The palatability test indicated a preference for the diets containing the protein product but the digestibility of protein and fat were reduced with increasing incorporation levels.

From these data it can be concluded that the inclusion of the protein product in the feeds of the above 4 animal species carries no appreciable risks for animal health, when added at a rate of up to 12% in the diet of calves weighing 110 kg but not at that level as milk protein replacer in calves at birth weight, up to 8% in the diet of pigs weighing 25 kg but not at that level in suckling pigs, up to 3% in the diet of broiler chicken, and up to 33% in that of salmon.

The statement, that in none of the feeding trials did any health problems, related to a disturbance in the microbial flora of the gut, arise is not substantiated by any experimental evidence.

Effects on the quality of animal products

As the protein product actually used as additive contains only normal feed ingredients, e.g. proteins, fat, carbohydrates and minerals, no toxic or other adverse residues are assumed to be present in the edible animal products. Tests for the presence of possible antibiotics and known toxins are carried out routinely on the added protein product. The organoleptic properties of the meat obtained from animals fed the protein product have been tested and found to be satisfactory.

Effects in laboratory animals

The nutritional studies provide evidence that this protein product is metabolized in the same way as conventional proteins.. Hence rigorous toxicological, metabolic and residue studies appear not to be meaningful.

No available data show that the bacterial strains used in production do not elaborate toxins and are not pathogenic. The analysis of the chemical composition of protein product has shown that nitrosamines are not present at the detection limit of 0.3ig/kg dry matter, and that methanol and polycyclic aromatic hydrocarbons are not present at the detection limit of 5 mg/kg dry matter. No significant toxicity was noted in the feeding studies in target animals.

A 4-week oral feeding study in rats was carried out using 5 groups of Wistar rats, each of 5 males and 5 females, fed a standard diet containing added 0%, 5%, 10% and 15% of the protein product. A positive control group received added 15% of a standard protein. Only a few incidental clinical changes were noted. Bodyweight gain of all groups was comparable to controls. There were no consistent differences between the groups regarding food consumption. Serum urea levels of males and females in the top test and positive control groups were increased. Serum creatinine levels in females of these groups were reduced. The relative kidney weights of the 10% and 15% (females only) test groups and the positive control group were increased. These changes were the expected consequences of high protein intake. Gross pathology and limited histopathology showed no treatment-related changes.

A 90-day oral feeding study in rats was carried out using 5 groups of Wistar rats, each of 10 males and 10 females, fed a standard diet with added 0%, 5%, 10% and 15% of the protein product, the positive control group receiving added 15% standard protein.

No adverse clinical symptoms related to treatment were noted. Bodyweight, food consumption, water consumption and food conversion ratios were comparable to controls. Haematology was unremarkable.

The two top test groups showed a small rise in serum alanineaminotransferase levels indicating slight hepatocellular dysfunction. Serum ornithine carbamyltransferase levels were unchanged and hepatic histology was normal. Serum urea levels were increased in the top test and positive control groups. Urinary excretion of N-acetyl-Â-D-glucosaminase was increased in the top test groups and in males of the 10% test and positive control groups without any associated renal histopathology. This slight leakage of renal tubular enzymes was probably due to the high protein load but an additional toxic effect of the protein product cannot be excluded. The increase in female relative kidney weights of the top dose were probably related to the higher bodyweight. Gross and histopathology showed no treatment-related findings.

A 90-day oral feeding study in minipigs was carried out in 5 groups, each of 4 male and 4 female minipigs, given a standard diet with added 0%, 5%, 10% and 15% of the protein product, the positive control group receiving a corresponding high-protein diet.

There were no adverse clinical signs and no toxicologically significant changes in bodyweight, food consumption, haematology, clinical chemistry, urinalysis and organ weights compared to controls. Gross pathology and histopathology showed no treatment-related changes.

No studies have been carried out in laboratory animals on multigeneration-reproduction, teratogenicity, chronic toxicity and carcinogenicity. However, no toxic effects were noted on the reproductive organs in the laboratory animals examined. The submitter has undertaken to carry out reproduction studies, if use of the protein product is to be extended to animals capable of reproduction.

The protein product was examined for genotoxicity in a salmonella reverse mutation test using strains TA98, TA100, TA1535 and TA1537 +/- S9 and dose levels varying fro 0.63mg to 10 mg/plate. No increase in revertants was found, thereby confirming absence of mutagenic activity in this system.

The protein product was also tested in a mouse micronucleus test at dose levels of 1.25 g, 2.5 g and 5 g/kg b.w. No significant increase in polychromatic erythrocytes with micronuclei was seen, thereby confirming absence of mutagenic potential in this test system.

The absence of significant toxicity including genotoxicity and the anticipated digestive breakdown of the protein product appear to indicate that it is toxicologically safe as protein source in animal feeds at the proposed levels. Further animal testing is not deemed necessary.

Protective measures against dust inhalation for the production workers and the users are recommended pending information to be provided as to the allergenic potential of this bioprotein.

Effects on man

Skin and eye irritation potential was examined in rabbits and showed that the protein product was non-irritant to the skin and eyes. The allergenic potential of the protein product has not been investigated.

Effects on the environment

The protein product contains no substrate residues or heavy metal contaminants except for a maximum copper content of 100 mg/kg, dry matter. It carried no viable production organisms because of the sterilisation step in the production of the final product. Any escape into the environment of the production organisms from the fermenter causes no hazard as these organisms all occur naturally, are non-pathogenic, and carry no virulence plasmids.

Monitoring in foodstuffs

The protein product can be determined in feedingstuffs qualitatively by indirect immunofluorescence using antibodies specific against *Methylococcus capsulatus* (Bath) and quantitatively by a spectrofluorimetric method with a sensitivity better than +/- 1%. Techniques to determine the proportions of each of the constituent organisms are not described in the dossier.

Conclusions

The protein product obtained from methylotrophic bacteria has been the subject of a basic dossier and two supplementary dossiers prepared in accordance with the guidelines for the assessment of certain products used in animal nutrition. This report is therefore limited to the assessment of this particular protein product, in the light of the information provided.

The product examined has an acceptable nutritional value as a source of protein for feeding to animals provided the inclusion levels in feeds do not exceed the values set out in the suggested conditions of use. It is not suitable for ewe milk replacers and further extensions should fill the remaining gaps in the general nutritional information.

On the base of the information provided by the Firm, the product examined carries no appreciable risks for livestock, if the levels of incorporation do not exceed 8% in the ration of pigs starting at weight 25 kg, does not exceed 8% in the feed of veal calves starting at weight 80 kg, does not exceed 19% in the feed of freshwater salmon and 33% in the feed of seawater salmon up to 3 years. There were no data to protein level of inclusion in the feed of piglets and non ruminant calves.

It poses no appreciable risk on presently available evidence for the health of workers involved in its production, distribution and use, if adequate precautions are taken to prevent exposure to dust. Because the allergenic potential of the bioprotein has not been investigated a warning should be included on the label, that the dust may cause sensitisation by inhalation and may give rise to respiratory allergic reactions in susceptible people.

It carries no microbiological risks because of its origin from non-pathogenic naturally occurring bacteria, known not to produce antibiotics or toxins and not being present as viable organisms in the final product because of its sterilisation.

It has no adverse toxicological or genotoxic effects although reproductive toxicology, teratogenicity, chronic toxicity and carcinogenicity have not been specifically investigated in laboratory animals, and is free from harmful contaminants arising from the culture medium or manufacturing process but can contain up to 100 mg/kg dry weight of copper, which would be diluted when mixed into composite feeding stuffs. Its use in animal feed does not result in appreciable risks for the environment.

It carries no appreciable risk for the consumer from the consumption of products obtained from animals fed with a diet containing this protein product. The characteristics and organoleptic properties of such animal products from chicken, pigs and salmon have been investigated and show no deleterious properties.

It can be monitored in feedstuffs, although no techniques are described to determine the proportions contributed by each of the constituent 4 bacteria to the biomass.

Suggested conditions of use

During the examination of the registration files it has been observed growth depression in some target animal species, and that, based upon the information provided by the firm, it exists a lack of data concerning the metabolisms of cyclopropanoics and other non identified fatty acids, that are present in the product and may be present in the animal product lipids.

Further to these observations, the Committee has judged that it will be prudent to limit the conditions of usage of this product as follows:

- Growing pigs from 25 kg up to a 100 kg live weight.

- The quantity of inclusion in complete feedingstuffs should no exceed
    - 8% for piglets starting at 25 kg
    - 8% for veal calves starting at 80 kg
    -19% for salmon fish in fresh water
    -33% for salmon fish in seawater

- The amount of protein provided by the product should be expressed as percentage of the total protein content of the compound feedingstuffs

These declarations are to be made on the label or packaging of the compound feedingstuffs, and should be introduced in the usage conditions requested by the company (See annexed table)

Future extension of use to other species or type of animals

If in the near future the firm asks for an extension of use to other species or type of animals providing edible products to the human consumer the following information should be provided.

For chickens for fattening, data concerning the availability of the individual aminoacids at different inclusion levels to be able to elucidate the cause and mechanisms of the fall in performances and sufficient results to assess the optimum inclusion level to exclude nutritional imbalances from an overdosage of the product in their diet. For pigs: more field trials are required

For all animals providing edible products to the consumer, analysis of the true content of cyclopropanoic acid in their lipids, instead of the expected content by calculation.

References

Dossiers on BioProtein*, Sections I-IV (1994) by (b) (4)
Pedersen A.-T., Skrede A., 011i J. & Eggebo L.M. (1994) Report submitted Oct. 15, 1994.
Skrede A., Herstad O., Sundstol F., Overland M. & Mroz, Z. (1994) Report submitted Oct. 15, 1994.
Supplement (VII) dossier by (b) (4)

Suggested conditions of use

1	2	3	4	5	6	7
Name of roduct group	Name of product	Designation of nutritive principle or identity of the micro-organism	Nutrient substrate Specifications if any	Composition characteristics of the product	Animal species	Special provisions
1. Bacteria						
1.2. Bacteria ıltivated on ıtural gas	1.1.2.1 Protein product of fermentation from natural gas obtained by culture of: *Methylococcus Capsulatus* (Bath) *Alcaligenes Acidovorans*, *Bacillus brevis*, *Bacillus*	*Methylococcus capsulatus* (Bath) NCIMB 11132, *Alcaligenes acidovorans* NCIMB 12387, *Bacillus brevis* NCIMB 13288, *Bacillus firmus* NCIMB 13280	Natural gas, 91% methane, 5,1% ethane 1,9% propane 0,4% isobutane 0,5% n-butane 1,1% other minor components), ammonia, mineral salts	Nitrogen expressed as crude protein minimum 65%	- Pigs from 25 kg - Calves from 80 - Salmon Fish.	Declaration to be made on the label or packaging of the product: - the name: "Protein product obtained by fer- - nitrogen expressed as crude protein - crude ash - crude fat - moisture content - instructions for use - declaration of the words "avoid inhalation" - The quantity of inclusion in complete - 8% for piglets starting at 25 kg - 8% for veal calves starrting at 80 kg - 19% for salmon fish in fresh water - 33% for salmon fish in sea water Declaration to be made on the label or packaging of the compound feedingstuffs:

	firmus, -the cells of which have been killed					- the name: "Protein product obtained by fer- - Amount of the product contained in the - Amount of protein provided by the product

Part B - Statement of Drs. Judith T. Zelikoff and Daniel Wierda

I. Statement Introduction

We have been requested by Calysta, Inc., to review relevant safety data, much of which is part of the pending GRAS notice submitted to FDA. We have reviewed these and other data (referenced below) and offer the following statements and conclusion.

By way of background, we understand from Calysta that the US Food and Drug Administration Center for Veterinary Medicine (CVM) expressed concern that elevation in serum IgG2a levels reported in mice[8] suggests the potential for long-term dietary exposures to FeedKind® (equivalent to Bioprotein) to pose a risk of chronic inflammation in salmonids.

Specifically, Christensen *et al.* (2003) characterized the serum and salivary antibody profiles of mice exposed to Bioprotein (BP) and nucleic-acid reduced Bioprotein (NRBP) in the diet. These investigators focused part of their study on IgA production, because of its association with gut immunity, and another part of their study on IgM, IgG1 and IgG2a production. Christensen *et al.* (2003) reported that the IgG1 response abated, but the IgG2a response was sustained in mice after the diet containing NRPB was replaced with the control diet. From this observation, we understand that CVM postulated a cause for concern that chronic feeding of FeedKind® to animals may be detrimental because IgG2a can be a marker for inflammation.

Extensive published chronic and sub-chronic exposure studies demonstrate that salmonids thrive on diets containing up to 18% or more BP, with no signs of inflammation or other adverse health effects that can be attributed to FeedKind®.[9] In addition, in other published studies, dietary BP antagonizes the well-known inflammatory responses to dietary soybean meal (SBM) observed in the gut of salmon.[10] Calysta concluded from these studies that FeedKind® does not produce adverse health effects in salmonids through an immunogenic mechanism. We understand that CVM responded that it would be acceptable to address their concern by providing a statement from the published literature confirming that the toxicity endpoints of the existing salmonid studies are appropriate for demonstrating no adverse health effects attributable to antibody-mediated (*i.e.* humoral) responses to FeedKind® in the diet.

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[8] Christensen HR, Larsen LC, Frøkær H (2003). The oral immunogenicity of BP, a bacterial single-cell protein, is affected by its particulate nature. Brit. J. Nutr., 90: 169-178.

[9] Berge GM, *et al*. (2005) Bacterial protein grown on natural gas as protein source in diets for Atlantic salmon, *Salmo salar*, in saltwater. *Aquaculture*. 244: 253-240; Aas TS, et al. (2006a) Improved growth and nutrient utilization in Atlantic salmon (Salmo salar) fed diets containing a bacterial protein meal. Aquaculture. 259: 365-376; Aas TS, *et al.*(2006b). Effects of diets containing a bacterial protein meal on growth and feed utilization in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*. 261: 357-368; Storebakken T, *et al.* (2004) Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo salar*, in freshwater. *Aquaculture*. 241: 413-425; See summaries of these studies in the GRAS Notice for Dried *Methylococcus capsulatus* Product (submitted to US FDA CVM on 2/28/2020)

[10] Romarheim OH, et al. (2011) Bacteria grown on natural gas prevent soybean meal-induced enteritis in Atlantic salmon. J Nutr. 141: 124-130; Romarheim OH, et al. (2012). Prevention of soya-induced enteritis in Atlantic salmon (Salmo salar) by bacteria grown on natural gas is dose dependent and related to epithelial MHC II reactivity and CD8α+ intraepithelial lymphocytes. Br J Nutr. 109 (6): 1062-1070.

Calysta uncovered no literature that explicitly addresses the concern that increased serum IgG2a levels or other humoral effects in animals fed diets containing protein products derived from bacteria or from other sources can cause inflammation. We are also aware of no such literature. Thus, Calysta solicited our opinion as independent specialists in the fields of fish and mammalian immunotoxicology to determine whether the data from the available studies supports a conclusion of a *reasonable certainty of no harm* to salmonids fed FeedKind® at the maximum intended use level of 18% in the diet. Our curriculum vitae are attached to this amendment.

We have reviewed the published rodent and salmonid studies, together with supporting unpublished studies, and conclude that the concern postulated by CVM is not valid and is not supported by the current scientific literature. Collectively, the studies we have reviewed and discussed below provide *reasonable certainty of no harm to salmonids* exposed to FeedKind® (equivalent to BP). We, therefore, conclude that based on the published studies as supported by the unpublished studies, FeedKind® is generally recognized as safe (GRAS) in the diet of salmonids at the intended use concentrations The published and unpublished studies are briefly summarized, below, followed by a discussion of the relevant information and rationale leading to the conclusion of this opinion.

II. Pertinent Rodent Studies

Christensen *et al.* (2003) fed mice for 56 days a diet containing one of the following: 24% casein (controls), 6% NRBP, 24% NRBP, or 24% BP. The NRBP was prepared by adding Fe_2SO_4 to BP and subjecting the mixture to heat-shock treatment to activate endogenous RNase and DNase, and then incubating at 60°C to allow the activated enzymes to degrade the nucleic acids. Christensen *et al.* (2003) found that BP-specific total Ig, IgA, IgG1, and IgG2a antibody titers in blood and BP-specific IgA antibody levels in saliva were significantly elevated in mice exposed to BP or to NRBP, compared with controls. No statistically significant differences were found in these parameters in the mice exposed to 24% BP compared with the mice exposed to 24% NRBP. The authors found that the treatment performed to reduce the nucleic acid content had no effect on the oral immunogenicity of BP, although homogenization to remove the particulate phase of the BP reduced the systemic immunogenicity of the compound.

An additional group of mice in the same study received 24% NRBP for 14 days followed by a control diet for 42 days. After cessation of exposure to NRBP, serum IgA levels declined precipitously to control levels by day 28, total serum Ig and IgG1 levels declined steadily to approximately 85% and 75%, respectively, by day 56, and IgA in saliva and Ig2a in serum remained at approximately 82% and 100%, respectively, by day 56. Christensen et al (2003) concluded that BP-specific IgA levels were induced solely at mucosal sites, without a systemic IgA response, and that the factors supporting a Th-2/IgG2A response may be more efficiently cleared from the immune system than the factors supporting a Th-1/IgG1 response. In mammals, Th1 cells are assumed to be associated with generation of IgG1. Christensen et al (2003) also suggested that prolonged exposure to BP or NRBP in the diet may result in the maintenance of a balance between the levels of IgG1 and IgG2A and, thus, between Th-1-type (pro-inflammatory) cytokines and Th-2-type (anti-inflammatory) cytokines. Such a balance could reduce or eliminate any risk of chronic inflammation that might exist in mammalian target

species fed diets containing BP.

Christensen *et al.* (2003) noted that the systemic antibody responses observed in their study in mice exposed to BP or NRBP may be related to the accumulation of foamy macrophages in the mesenteric lymph nodes (MLNs) as reported by Mølck *et al.* (2002) in rats exposed NRBP in the diet for 90 days.[11] Related effects were reported by Glerup (1999)[12] in rats receiving BP in the diet for 56 days. However, these and other sub-chronic oral exposure studies demonstrated that BP levels up to and including 15% in the diet of rats yielded minimal-to-no evidence of inflammation in the intestines or other organs examined and no signs of endotoxemia.[13]

Accordingly, Glerup (2002) reported similar results in an unpublished study in rats receiving up to and including 22% Brewer's yeast for 28 days. Christensen *et al.* (2004)[14] reported that mice exposed to soy protein in the diet or in drinking water across 3 generations (F_0, F_1, and F_2) exhibited antibody responses coinciding with the induction of oral tolerance, and that these appear to be commonly-seen responses to the ingestion of soy protein. The immunogenicity of orally administered BP in mice may be analogous to those of Brewer's yeast, soy protein and/or other protein sources commonly used in animal feeds, including the induction of oral tolerance.[15]

Thestrup (2004)[16] evaluated the serum antibody data from two unpublished studies in which rats received BP or Brewer's yeast in the diet. The studies included a one-generation reproductive toxicity study[17] in which the animals were fed a diet containing 6% or 12% BP or Brewer's yeast, as well as a 90-day study[18] in which juvenile rats were fed a diet containing 12% BP.[19]

The oral exposures in these studies produced elevation of BP-specific serum levels of IgA, IgG, IgG1, and IgG2Aa (ranging from 1-fold for IgA to 3-fold for IgG1 compared with

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[11] Mølck A-M, Poulsen M, Christensen HR, Lauridsen ST, Madsen C (2002). Immunotoxicity of nucleic acid reduced BP – a bacterial derived single cell protein – in Wistar rats. Toxicology 174: 183-200.

[12] Glerup P (1999). Eight-week lymph node toxicity study in the rat. Scantox test report, prepared for Dansk BP A/S, Lab No. 30864, 20 September, 73 pp.

[13] Glerup P (1999). Eight-week lymph node toxicity study in the rat. Scantox test report, prepared for Dansk BP A/S, Lab No. 30864, 20 September, 73 pp; Svendsen O, Damm-Jørgensen K (1992). Single cell protein: three-month oral toxicity study in the rat. Scantox test report, prepared for Dansk BP A/S, Lab. No. 12960, July 31, 91 pp; Takawale P (2004). BP: Study in juvenile rats. Scantox test report, prepared for Norferm A/S, Study no. 52692, 20 October, 166 pp; Thestrup HN (2004). BP antibody responses in feeding studies. Internal report, Norferm Denmark, 14 October, 12 pp.

[14] Christensen HR, Brix S, Frøkær H (2004). Immune response in mice to ingested soya protein: antibody: antibody production, oral tolerance, and maternal barrier. Brit. J. Nutr., 91: 725-732.

[15] For example, see: https://www feedipedia.org/node/72; https://feedipedia.org/node/674.

[16] Thestrup HN (2004). BP antibody responses in feeding studies. Internal report, Norferm Denmark, 14 October, 12 pp.

[17] Takawale P (2004). BP: Study in juvenile rats. Scantox test report, prepared for Norferm A/S, Study no. 52692, 20 October, 166 pp.

[18] Clausing and Bøgh (2002). BP: One generation reproduction toxicity study in rat. Scantox test report, prepared for Norferm A/S, Lab No. 25995, 22 January, 263 pp.

[19] See also Noferm AS (2004). Supplement to BP® Dossier. Submitted for registration of BP® in the EU under directive 82/471/EEC.

controls). The antibody responses were one to two orders of magnitude weaker than the responses observed in control rats challenged with BP by *i.p.* injection. In addition, the antibody responses to orally administered BP were elevated in the parental animals, but not in the offspring. In the rats exposed to Brewer's yeast, serum IgA, IgG1, and IgG2a levels specific for Brewer's yeast produced essentially the same pattern of antibody responses as the BP-specific antibody levels observed in the rats exposed to BP.

In the 90-day study, the antibody responses in rats fed BP beginning at 3-weeks-of-age were significantly lower than those observed in rats fed BP beginning at 7 weeks of age.

As noted by Thestrup (2004), the results of the one-generation study and the 90-day study in rats, taken together, could indicate that immunological tolerance to BP was induced in rats exposed orally to BP. This interpretation is consistent with the results of other studies reporting minimal-to-no evidence of inflammation in the intestines or other organs of rats receiving BP in the diet.

In other experiments, Christensen *et al.* (2003) showed that spleen cells from mice exposed to NRBP exhibited a statistically significant increase in splenocyte (lymphocyte) proliferation, indicating that T-lymphocytes were activated in the exposed animals, and that the mucosal immunogenicity, but not the systemic immunogenicity, of the NRBP was retained in a whole-cell-free BP homogenate, implying that the particulate nature of BP plays a crucial role in the systemic immunogenicity of ingested BP. The latter effects can be attributed to the more efficient transfer of larger particles from the mesenteric lymph nodes (MLNs) to the spleen, which could elicit systemic IgG responses, compared with smaller particles in the digestive tract. The bacterial cells associated with BP are optimum in size for partial systemic translocation through the MLNs and partial retention in the Peyer's patches of the digestive tract of mammalian species, which helps to explain why BP could induce a systemic as well as a mucosal immune response in mammals.

However, these results are not inconsistent with the reported absence of inflammatory responses attributable to BP in rats exposed to BP in the diet. As Christensen *et al.* (2003) noted, the lipopolysaccharides (LPS) of the cell membrane of *Methylococcus capsulatus* may be responsible for the adjuvant component of BP. They also noted that the mucosal adjuvant activity of LPS is quite complex; could enhance oral tolerance to antigens; and varies substantially from one bacterial species to another in their effects on antigen presenting cells.

III. Pertinent Salmonid Studies

Berge *et al.* (2005) fed groups of saltwater-maintained Atlantic salmon a diet of 0%, 10%, or 20% BP for 5 months.[20] Histological examinations indicated that the mucosa of the distal intestines was generally normal across all of the groups, including the numbers of absorptive vacuoles in the enterocytes of the intestinal folds and amounts of leucocytes infiltrating the mucosa and submucosa. One fish in the 10% BP group exhibited a severely

[20] Berge GM, Bæverfjord G, Skrede A, Storebakken T (2005). Bacterial protein grown on natural gas in diets for Atlantic salmon, Salmo salar, in saltwater. Aquaculture, 244: 233-240.

inflamed intestinal mucosa, but the body weight and length of this fish was close to the tank means and there were no external signs of disease. The authors concluded in their published study that there were no signs of any allergic-like reaction to BP, even in fish exposed to 20% BPM in the diet for 5 months.

Aas *et al.* (2006a) fed Atlantic salmon BP in the diet for 48 days.[21] The authors attributed the absence of adverse effects on mortality, growth rates and other indices of health in the salmon exposed to up to 36% BP in this peer-reviewed published study to improved utilization of the feed containing BP.

Aas *et al.* (2006b) fed rainbow trout 0%, 9%, 18%, or 27% BP or 9% BP autolysate in the diet for 71 days.[22] There were no statistically significant differences across the groups in mean body weight, specific growth rate (SGR), feed intake, or feed efficiency ratio (FER), or liver- or viscera-to-body-weight ratio at the end of the exposure period. Histopathological examinations revealed no exposure-related changes in morphology in any region of the gastrointestinal tract of the fish receiving up to 36% BP. In addition, there were no significant differences observed between fish fed autolyzed BP and those fed BP.

Romarheim *et al.* (2011) fed juvenile Atlantic salmon for 80 days a control high-quality fish meal (FM) diet or a diet in which the FM was incrementally replaced to contain 20% solvent-extracted soybean meal (SBM), 30% BP, or 20% SBM plus 30% BP.[23] Morphological, morphometric, and immunohistochemistry examinations in this published study revealed normal intestinal tissue in salmon receiving FM alone, BP, or SBM plus BP in the diet. In contrast, salmon fed diets containing SBM without BP exhibited SBM-induced enteritis in the distal intestine, including atrophy of simple and complex folds, loss of epithelial vacuolation, decreased epithelial-cell height, and prominent inflammatory-cell infiltration of the mucosa. Staining for proliferating cell nuclear antigen (PCNA, a marker of cell proliferation) revealed that the length of the stained sections of the distal intestines decreased in fish fed (in descending order):

(1) 20% SMB plus 40% FM
(2) 20% SMB plus 30% BP plus 10% FM
(3) 30% BP plus 30% FM
(4) 60% FM alone

A brush border reaction for carbonic anhydrase 12 (CA12) was conspicuously absent in the fish fed 20% SBM plus 40% FM, in contrast to the normal reactivity expressed in fish fed any one of the other diets.

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[21] Aas TS, Grisdale-Helland B, Terjesen BF, Helland SJ (2006a). Improved growth and nutrient utilization in Atlantic salmon (Salmo salar) fed diets containing a bacterial protein meal. Aquaculture 259: 365-376.

[22] Aas TS, Hatlen B, Grisdale-Helland B, Terjsen BF, Bakke-McKellep AM, Helland SJ (2006b). Effects of diets containing bacterial protein meal on growth and feed utilasation in rainbow trout (Oncorhynchus mykiss). Aquaculture 261: 357-368.

[23] Romarheim OH, Øverland M, Mydland LT, Skrede A, Landsverk T (2011) Bacteria grown on natural gas prevent soybean meal-induced enteritis in Atlantic salmon. J Nutr. 141: 124-130.

Romarheim *et al.* (2011) reported in another published study that fish fed 20% SBM in the diet exhibited significantly reduced final body weight, thermal growth coefficient (TGC), and feed conversion ratio (FCR) compared to fish fed the FM control diet. In contrast, 30% BP resulted in a statistically significant increase in FCR, but no statistically significant differences in final body weight or TGC. The authors concluded that the addition of BP counteracts or neutralizes SBM-induced enteritis in Atlantic salmon.

Romarheim *et al.* (2013) fed juvenile Atlantic salmon for 47 days a control FM diet or a diet in which the FM was incrementally replaced to contain 20% SBM plus 0%, 2.5%, 5%, 10%, 15%, 20% or 30% BP.[24] Enteritis was observed in all fish fed the diet containing 20% SBM without BP, which was reflected by accumulation of leucocytes in the lamina propria and widening of the lamina propria and submucosa that was attributable to cellular infiltration and edema, among other pathologies. These morphological changes decreased with increasing concentration of BP in the 20% SBM diet, with no evidence of enteritis in the fish receiving 20% or 30% BP with 20% SBM.

Romarheim *et al.* (2013) found that $CD8\alpha^+$ lymphocytes were prevalent at the base of the intestinal epithelium in salmon receiving a diet containing 20% SBM without BP, indicating that SBM-induced enteritis is a T-cell-mediated inflammatory response. Like the morphological changes, the mobilization of $CD8\alpha^+$ lymphocytes decreased with increasing concentration of BP, with no significant difference in the density of $CD8\alpha^+$ intraepithelial lymphocytes in fish receiving 20% or 30% BP in the diet containing 20% SBM, compared with the FM-fed controls. Intense staining for MHC-2 in the leukocytes at the base of the intestinal epithelium was indistinguishable from controls in fish fed 20% SBM plus 30% BP, unlike the staining observed in the fish fed the other diets containing SBM. The lengths of stretches of PCNA-stained sections of the distal intestines of the salmon fed 20% SBM decreased with increasing BP concentration and were indistinguishable from controls in the fish receiving ≥15% BP in the 20% SBM diet.

Romerheim *et al.* (2013) suggested in this same study that the most likely mechanism by which BP counteracts pro-inflammatory responses in salmonids exposed to 20% SBM in the diet is related to immune-system mechanisms that are also responsible for ensuring tolerance to feed antigens and to commensal intestinal microbiota. They noted that regulatory and CD8+ and CD4+ T-lymphocytes that express the Foxp3 transcription factor are known to play a key role in the prevention of inflammatory responses to food antigens and commensal bacteria in mice and in humans.

IV. Discussion

The immune system of fish has been extensively studied in only a few species, including

[24] Romarheim OH, Hetland D, Skrede A, Øverland M, Mydland LT, Skrede A, Landsverk T (2013). Prevention of soya-induced enteritis in Atlantic salmon (Salmo salar) by bacteria grown on natural gas is dose dependent and related to epithelial MHC II reactivity and CD8α+ intraepithelial lymphocytes. Br J Nutr. 109 (6): 1062-1070.

salmon, trout, and zebrafish.[25] In general, these studies show that fish share the basic components of the immune system with all other jaw vertebrates. However, there are known differences in the immune systems of fish, compared with those of mammalian species, as well as a plethora of unknowns in fish immunology. One major difference between bony fish and mammalian species resides in the adaptive, humoral arm of the immune response. The 3 major antibody/immunoglobulin types identified to date in teleost fish (depending on the species) are IgM, IgT/Z, and IgD as compared to the 5 classes (and several sub-classes) in mammals (*i.e.* IgG, IgD, IgM, IgE, IgA) each with distinct effector type functions. IgM constitutes the main systemic immunoglobulin in fish, IgT plays a key role in mucosal surfaces, and the role of IgD in fish immunity remains to be fully elucidated. Along with the lack of an IgG isotype, bony fish also lack Ig class switching recombination (Barreto et al., 2005).

CVM postulated a cause for concern that chronic feeding of FeedKind® to animals may be detrimental because IgG2a (as a result of IgG galactosylation) can be used as a biomarker for predicting inflammation.[26] While this is true for mammals, the evidence does not support this for bony fish. The widely accepted consensus of many research groups in this field is that the proinflammatory activity of IgG2 in mammals depends predominantly upon the presence of cellular Fc receptors (*e.g.*, FcγRs). Blockage of these receptors minimizes antibody-mediated inflammation.[27] Activated complement component C5a modulates the expression of FcγRs. Mice deficient in the C5a-receptor demonstrate a loss of antibody-mediated inflammation. In comparison, relatively few FcRs are found in fishes. In addition, fish have both teleost-specific receptor families (*i.e.*, novel immune-type receptors, NITRs) and receptor families that are distantly related to various mammalian immunoregulatory receptors belonging to the immunoglobulin superfamily (IgSF) (*i.e.* leukocyte immune-type receptors, LITRs), which presents a formidable challenge for determining the precise roles of all of the receptor-types in teleost immunity.[28] Furthermore, the complement system in bony fish, which is essential in mammals for Ig-mediated inflammation, is similar in many ways, but also quite distinct from that of mammals, and may not act in a way that is similar to the complement-activation/opsonization system of mammals.[29] Thus, from a mechanistic perspective it cannot be concluded that increases in serum IgG2 levels in mammals, or a possible equivalent in fish, will (or even could) lead to an inflammatory response.

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[25] For review see, for example, Magadan S, Sunyer OJ, Boudinot P (2015). Unique features of fish immune repertoires: particularities of adaptive immunity within the largest group of vertebrates. Results Probl. Cell. Differ. 57: 235-264. Barreto VM, Pan-Hammarstrom Q, Zhao Y, Hammarstrom L, Misulovin Z, Nussenzweig MC. (2005). AID from bony fish catalyzes switch class recombination. J. Exp. Med. 202(6): 733-738.

[26] Plomp R, Ruhaak LR., Uh H-W, Reiding KR, Selman M, Houwing-Duistermatt JJ, Slagboom PE, Beekman M, Wuhrer M. (2017). Subclass-specific IgG glycosylation is associated with markers of inflammation and metabolic health. Scientific Reports. 7:12325.

[27] Aschermann S, Lux A, Baerenwaldt A, Biburger M, Nimmerjahn F. 2010. The other side of immunoglobulin G: suppressor of inflammation. Clin. Exper. Immunol. 160(2): 161–167.

[28] Fei C, Pemberton JG, Lillico DME, Zwozdesky MW, Stafford JL. Biochemical and Functional Insights into the Integrated Regulation of Innate Immune Cell Responses by Teleost Leukocyte Immune-Type Receptors. Biology (Basil). Mar; 5(1): 13.

[29] Nonaka M, Smith SL. (2000). Complement system of bony and cartilaginous fish. Fish Shellfish Immunol. 2000 Apr;10(3):215-28.

As noted by Magadan *et al.* (2015), fish have the most extensive and complex mucosal surfaces among vertebrates, which include the skin as well as the gills and gut mucosa, the immunities of which are poorly understood. However, the protection of mucosa in fish appears to primarily involve IgT, which is analogous to the role played by IgA in mammals. Furthermore, fish mount protective immune responses despite the lack of lymph nodes and germinal centers that initiate immune responses in mammals. Instead, adaptive responses and T-/B-cell cooperation probably take place in the spleen of fish. Fish also lack Peyer's patches or similar encapsulated structures found in the gastrointestinal tract mammals. Thus, the gut associated lymphocyte tissue (GALT) simply comprise the macrophages, B and T lymphocytes and granulocytes of the digestive tract in fish. These substantial differences in the immune systems of fish species compared with mammalian species indicate that the mechanisms resulting in the systemic immune responses to dietary BP in mice and rats are not likely mechanisms associated with fish. For example, the absence of MLNs or analogous tissues in fish indicates that none of the particulates of BP in the gastrointestinal tract of fish can migrate to the spleen of the fish to stimulate a systemic immune system response.

It is also important to distinguish between the direct effects of a test substance on the immune system, which is the study of immunotoxicology, from immunological responses to the test substance that can cause inflammation and other indirect adverse health effects, which is the subject of this opinion.[30] The former, direct effects result from direct interactions of the test substance with molecules or cells of the immune system that lead to immunomodulation, immunoenhancement, or immunosuppression that lead, in turn, to subsequent adverse health effects. The latter, indirect effects result from the immunogenicity of the test substance.

The results of the studies summarized in the preceding sections, in which salmonids were exposed for up to 5 months to diets containing up to 36% BP, were consistently negative for signs of adverse inflammatory effects that can be attributed to FeedKind®. In salmonids, FeedKind® did not produce the exposure-related inflammatory responses that were suggested as possible based on the changes in the IgG2a titers in mice reported by Christensen *et al.* (2003). This conclusion is supported by unpublished studies in which salmon were fed diets containing up to 37% FeedKind® for up to 364 days without affecting body weight or other toxicity endpoints indicative of adverse health effects.[31]

The findings reported by Romarheim *et al.* (2012, 2013) provide substantial support for this conclusion. Conventional solvent-extracted SBM is considered a suitable protein source for farmed salmonids, although dietary inclusion levels as low as 7.6% are known to cause SBM-enteritis (characterized by inflammation of the distal intestines) in salmon. The mechanism for this reversible effect appears to involve impaired immune tolerance to SBM caused by alcohol-soluble components of SBM, such as saponins. In their published review, Martin *et al.* (2017) noted that SBM is now commonly used as a model for inducing gut inflammation (*i.e.* enteritis

[30] For review, see Rehberger K, Werner I, Hitzfeld B, Segner H, Baumann L (2017). 20 Years of fish immunotoxicology – what we know and where we are. Crit. Rev. Toxicol. 47(6): 516-542.

[31] Storebakken T, Bæverfjord G, Skrede A, Olli JJ, Berge GM (2004). Bacterial protein grown on natural gas in diets for Atlantic salmon, *Salmo solar*, in freshwater. Aquaculture. 241: 413-425.

and associated histopathological changes in the intestines).[32] Romarheim *et al.* (2011, 2013) showed that BP effectively counteracted the severe pro-inflammatory responses to 20% SBM in the intestinal mucosa of these fish, such that the responses were completely abated in the fish fed diets containing 20% SBM plus 15%, 20%, or 30% BP.

IgG2a is an implied biomarker for the immunogenicity of FeedKind® in *mammalian species*, as reported by Christensen *et al.* (2003). Bacterial antigens, which are present in feed ingredients derived from bacteria, are known to stimulate the production of IgG2a and other antibodies in mammals because bacteria stimulate toll-receptors on lymphocytes, which can induce Th-1-type responses. These responses include immunoglobulin class switching in B cells to produce IgG2a antibodies that optimize the clearance of extracellular bacteria and viruses.

However, stimulating antibody production, *per se*, does not mean that there will be pathological consequences to the host. For example, antibodies produced against the bacterial antigens and the adjuvants of orally administered vaccines do not cause host pathologies. The *i.m.* or *s.c.* injection of such vaccines typically cause no more than temporary inflammation at the injection site, which is not antibody-mediated. For instance, the production of IgG2a antibodies in mammals immunized parenterally against *Salmonella typhimurium* is stimulated through a Th-1 response. Likewise, anti-drug antibodies generated against monoclonal-antibody therapeutic agents counteract the pharmacological activity of the agents and remain in the patients in the long term, but generally do not cause any adverse health effects in the patients.

Accordingly, an increase in IgG2a in mammals is not a necessary corollary or an indicator of adverse inflammatory processes, including tissue damage, especially in bony fish (see information above).

As noted above, Christensen *et al.* (2004) found that IgG2a was produced in mice fed SBM. It is possible that antibodies analogous to IgG2a were produced in fish fed a diet containing SBM plus BP, as well as in the fish fed diets containing SBM without BP, in the studies by Romarheim *et al.* (2011, 2013). Nevertheless, the morphological, morphometric, and immunohistochemistry data revealed normal intestinal tissue in the salmon receiving the diet containing BP or the diet containing SBM plus BP, as well as in fish receiving the FM control diet, in contrast to fish fed a diet containing SMB without BP. This indicates that the postulated humoral immune-system response in fish fed diets containing BP, is likely not analogous to the production of IgG2A reported in mice by Christensen *et al.* (2003) and does not appear to be associated with consequent adverse inflammatory processes in the fish.

The studies summarized in the preceding sections show clearly that both BP and Brewer's yeast cause similar age- and sex-specific immune responses in fish and rodents. The humoral responses in fish are reflected in the changes in Ig levels and histological parameters observed in the spleen, which is considered to be the counterpart to mammalian lymph nodes. However, the outcomes of salmon growth rate and body weight measurements and intestinal inflammation studies indicate no overall toxicity. Thus, there is no indication that humoral

[32] Martin AM, Król E (2017). Nutrogenomics and immune function in fish: new insights from omics technologies. Develop. Compar. Immunol. 79: 86-98.

responses activated by BP (or Brewer's yeast), including the changes in Ig levels, lead to adverse effects or Ig-mediated inflammation.

V. Conclusion

Review of the published rodent and salmonid studies, together with the supporting unpublished studies and acknowledgement of mechanism differences between the mammalian immune response and that of bony fish, results in the conclusion that the concern postulated by CVM is not supported by the current scientific literature and that the studies provide *reasonable certainty of no harm to salmonids* exposed to FeedKind® (equivalent to BP). Thus, we conclude that that this ingredient is GRAS at the intended use concentrations up to a maximum of 18% in the diet.

Respectfully submitted,

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Signature Date

Judith T. Zelikoff, M.S., Ph.D.
Tenured Professor
NYU Grossman School of Medicine
Dept. Environmental Medicine
341 E. 25th Street
NY, NY 10010
Judith.zelikoff@nyumc.org
Office: 646-754-9451

(b) (6) 16 Jul 2020

Signature Date

Daniel Wierda, M.S., Ph.D., Fellow ATS
Wierda Toxicology Consulting, Inc.
2636 S Hillview Drive,
New Palestine, IN 46163 USA
drwierda@gmail.com
Office: 317-622-2079; Cell: 317-318-5720

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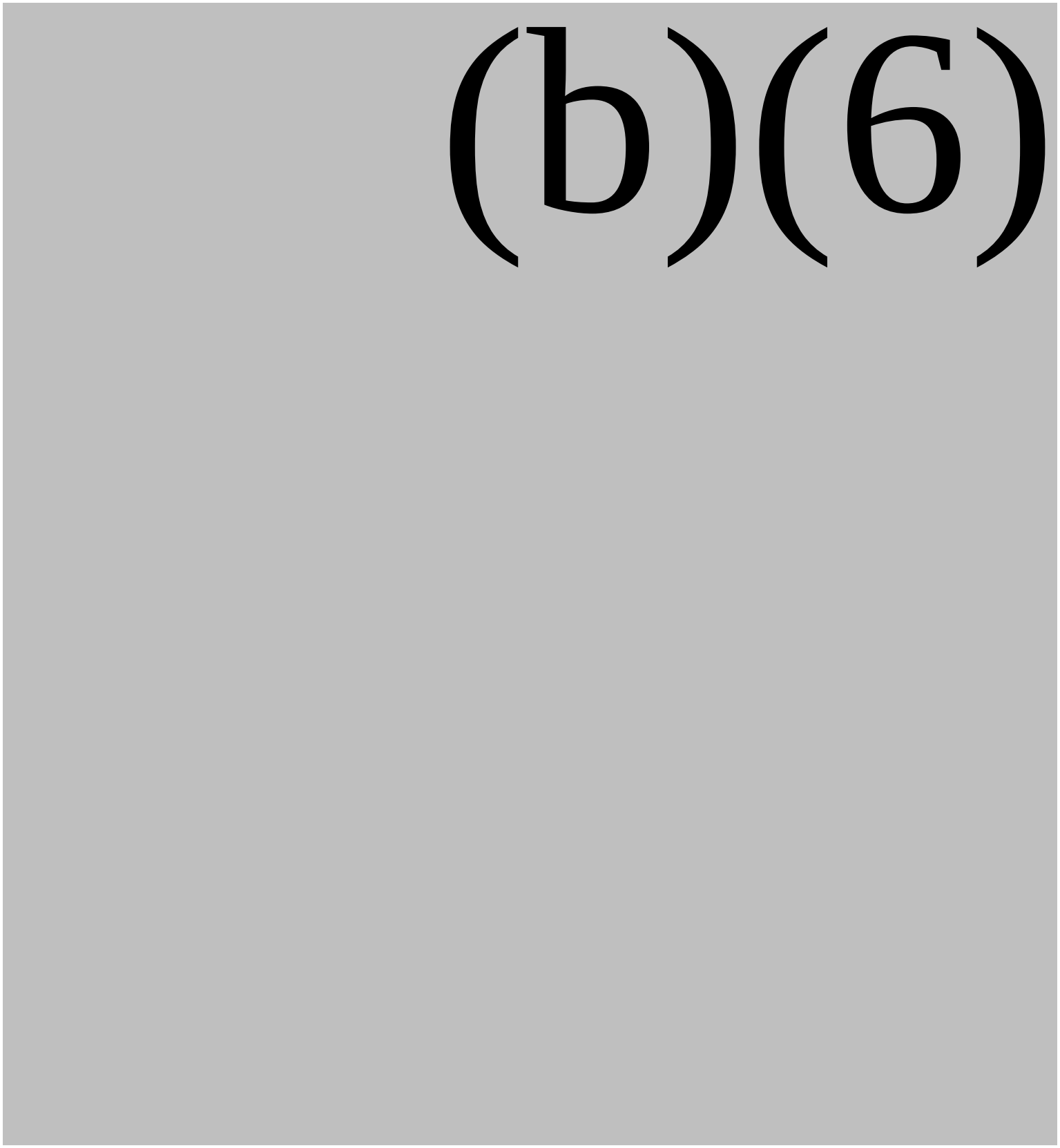
Expert Curriculum Vitae

JUDITH TERRY ZELIKOFF, Ph.D.
Tenured Professor, NYU Grossman School of Medicine; Dept. Environmental Medicine



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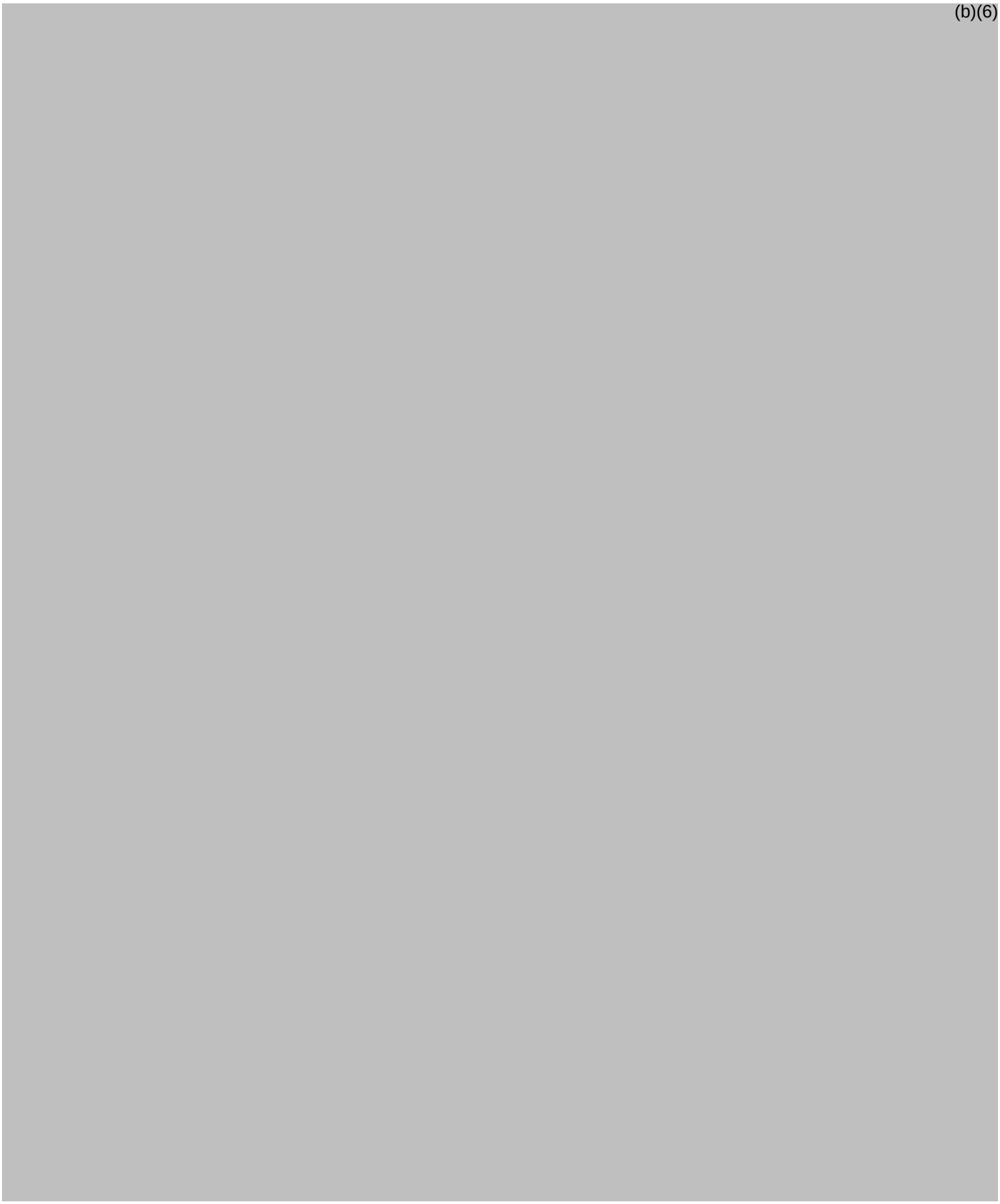

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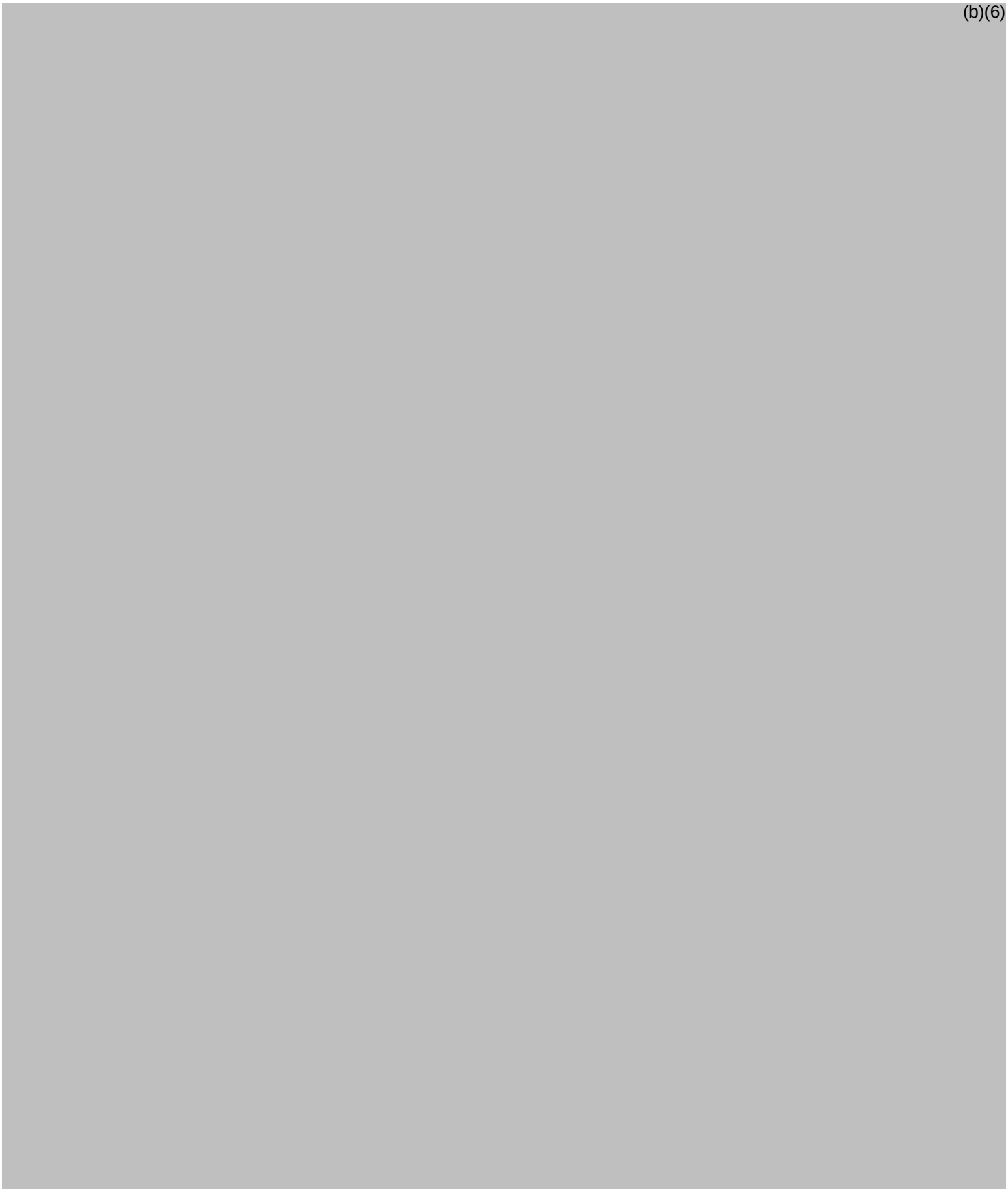

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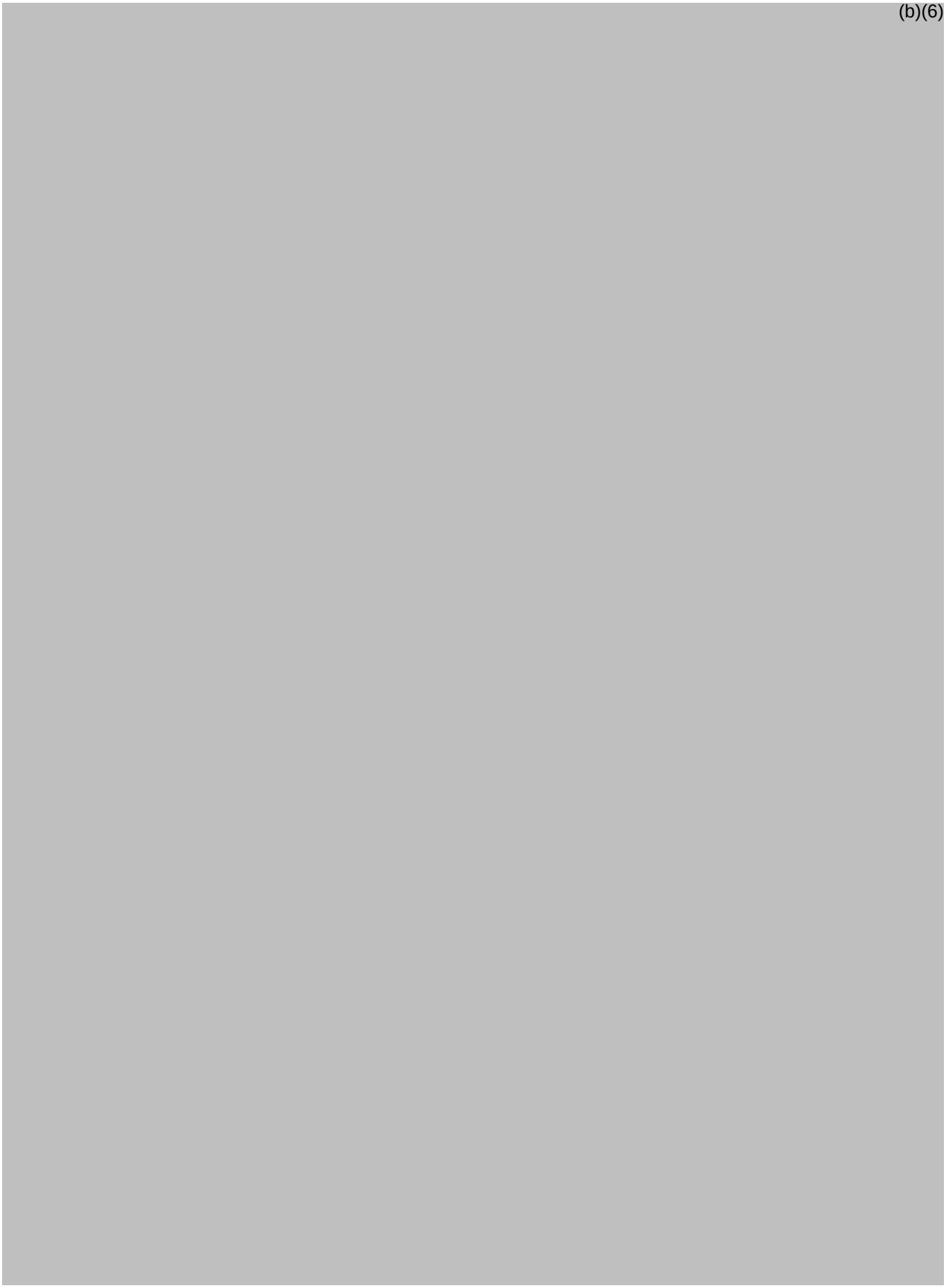

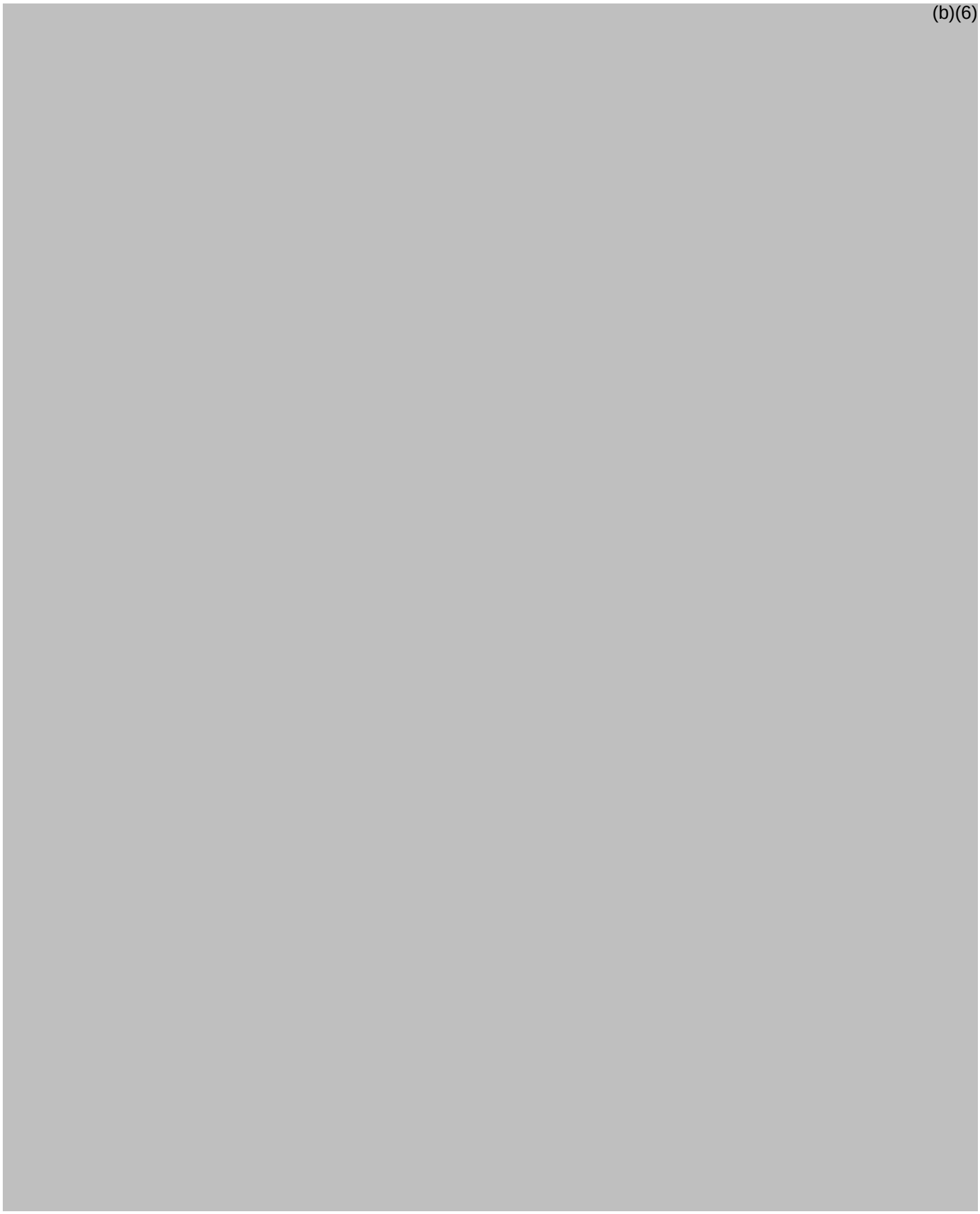
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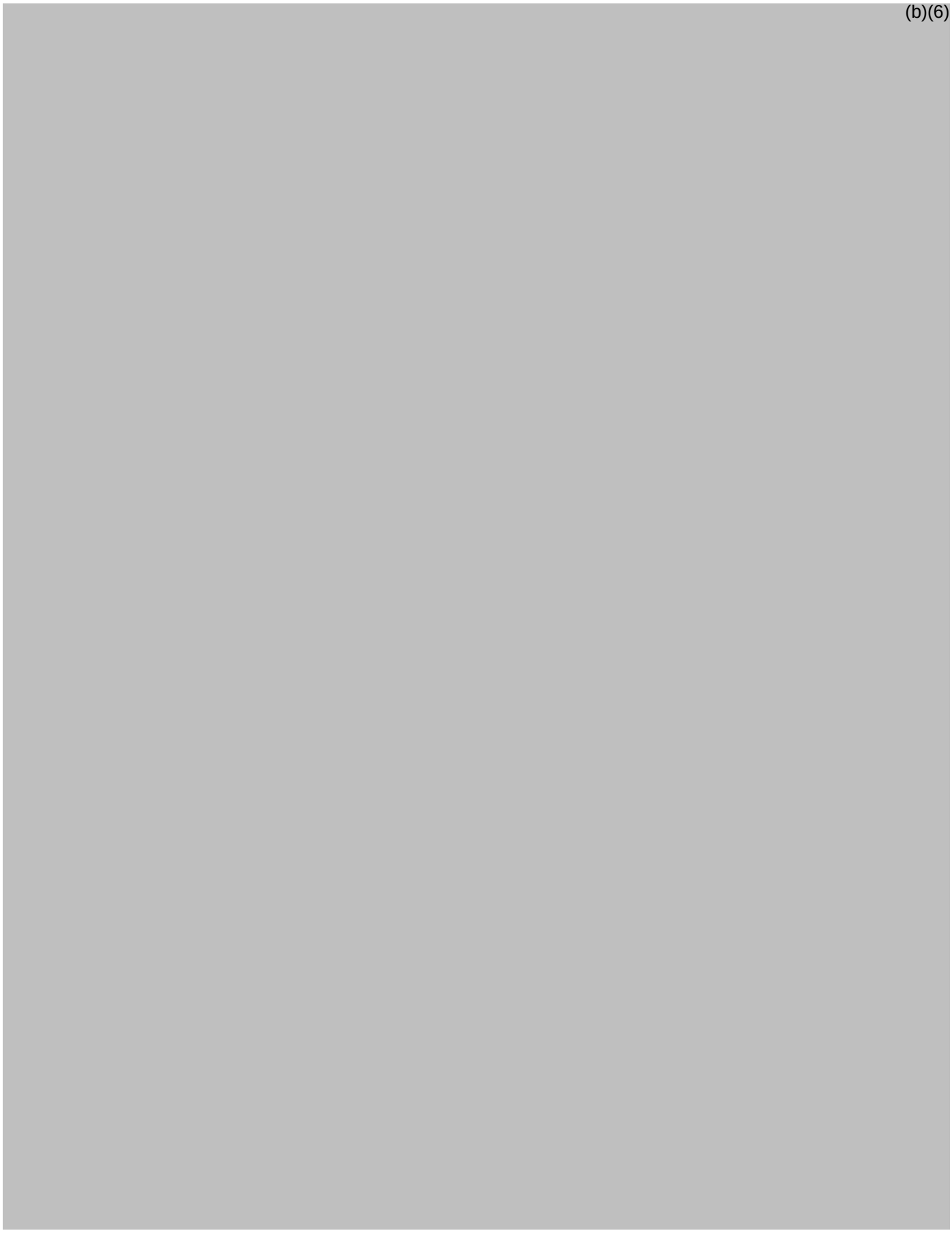
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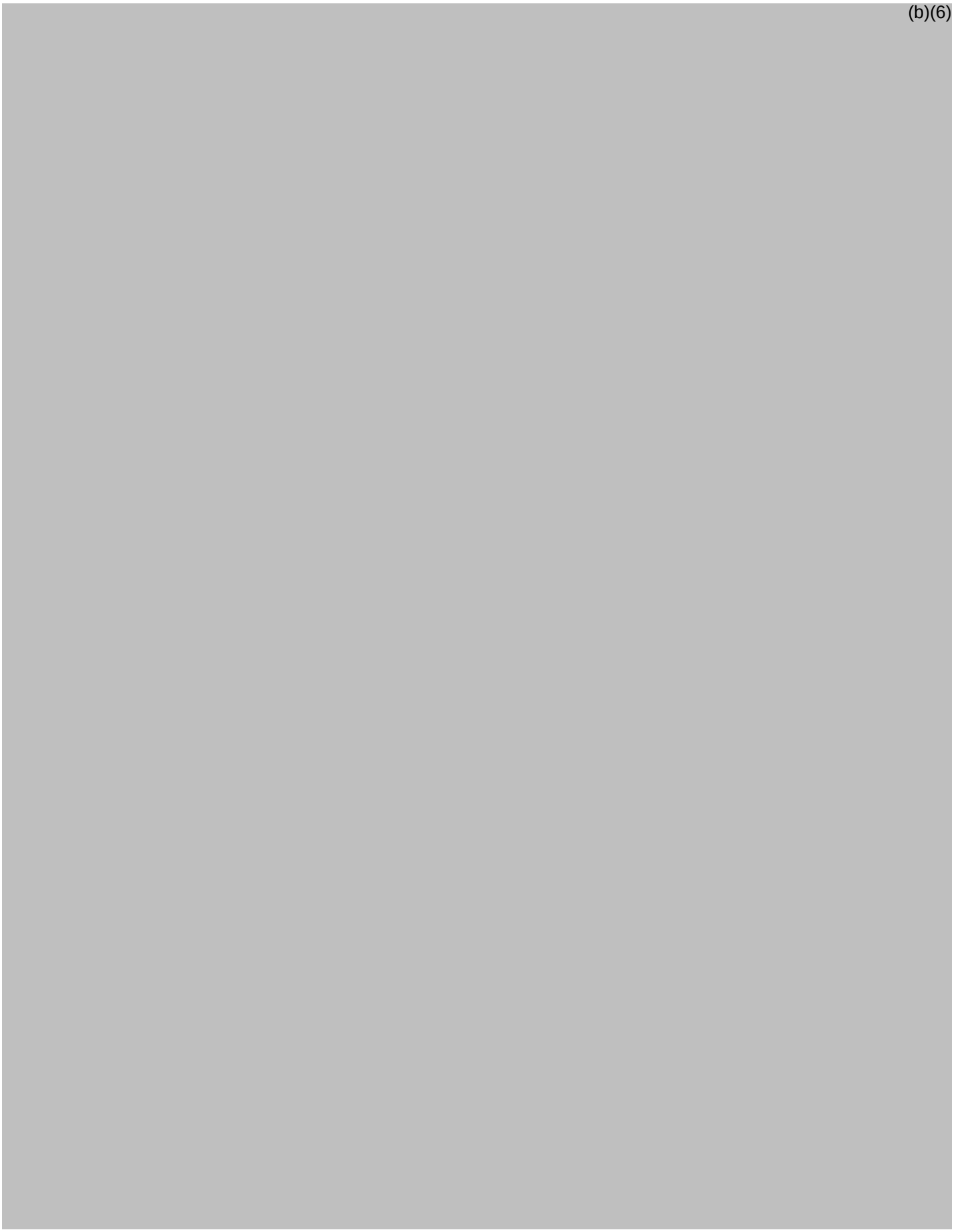
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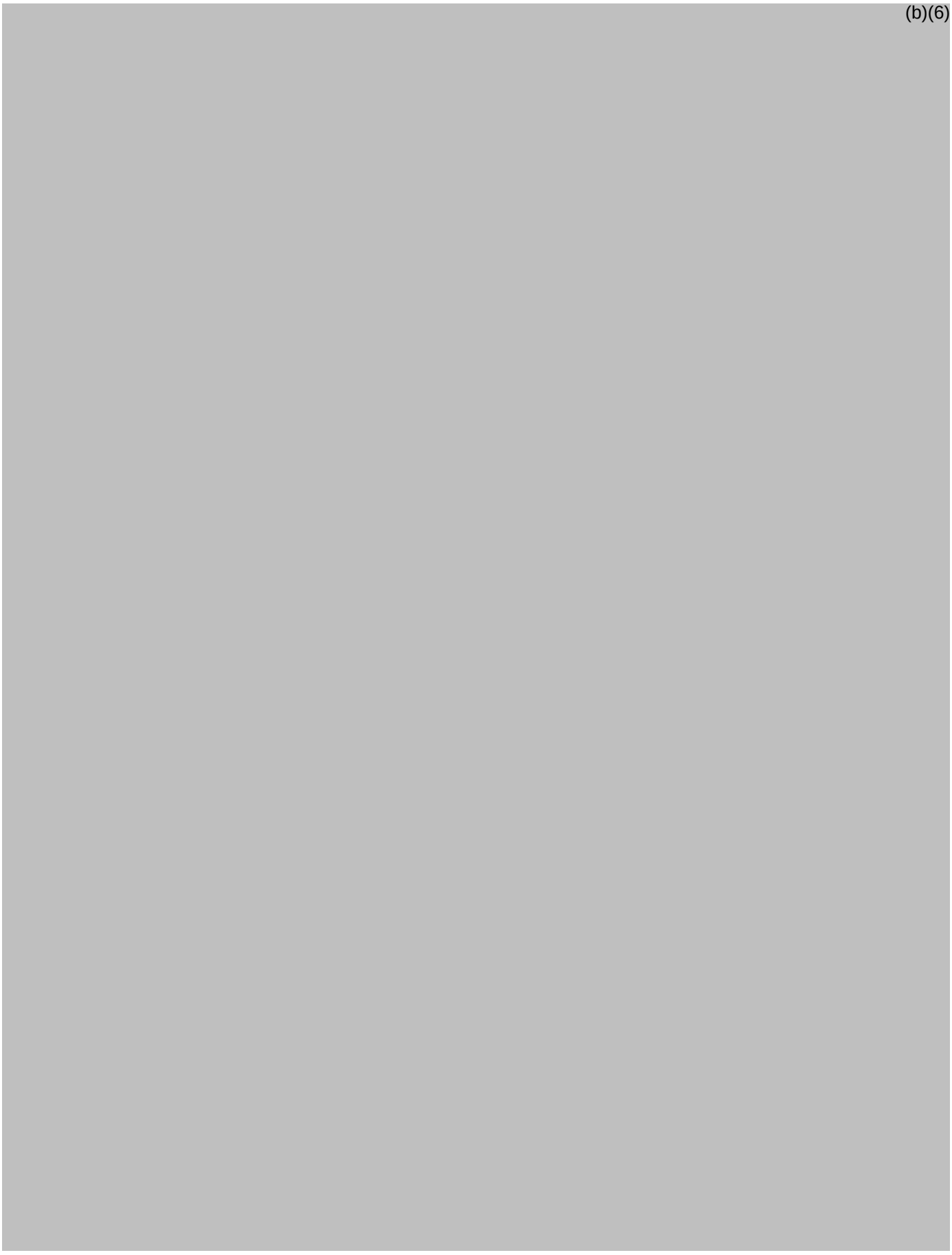

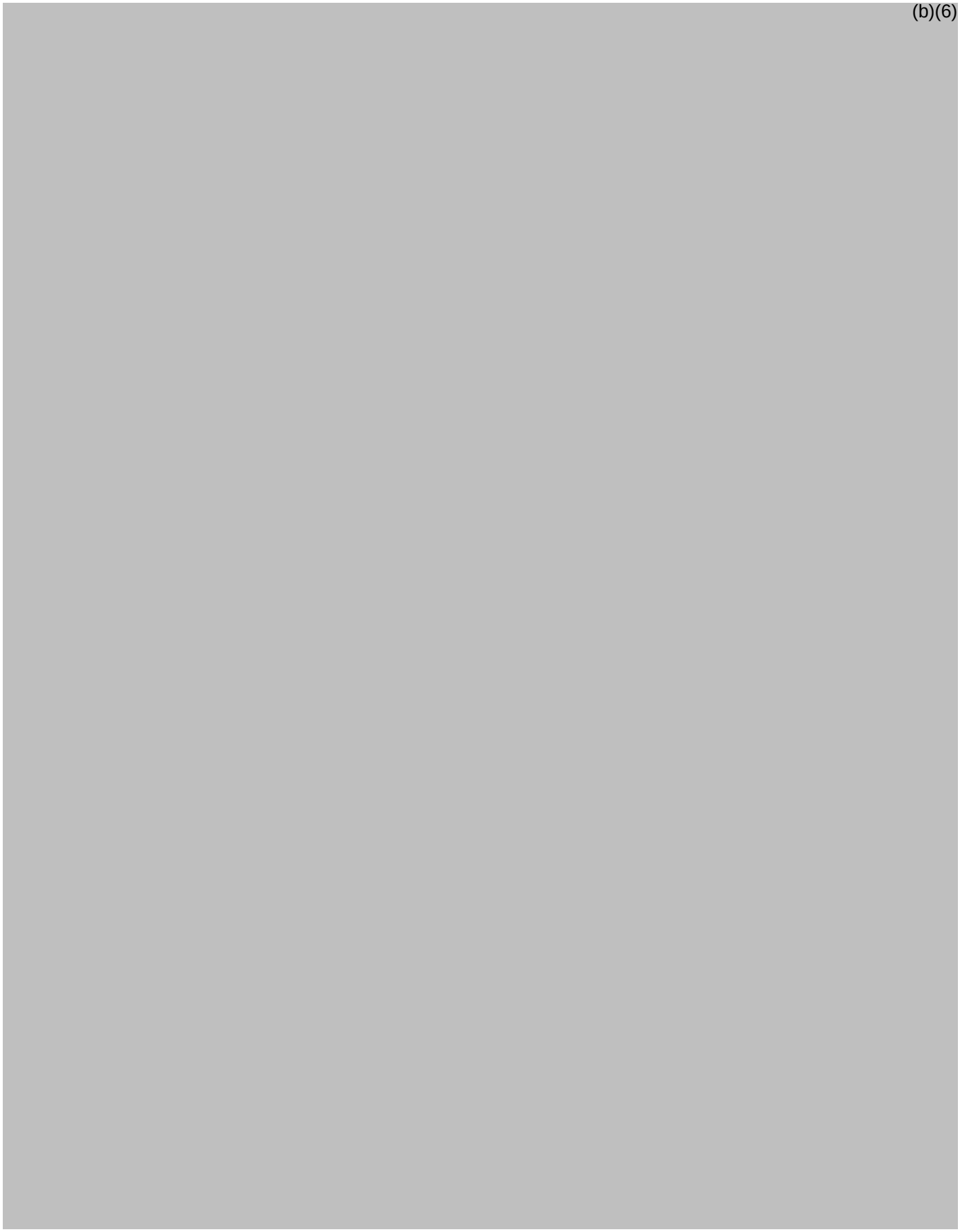
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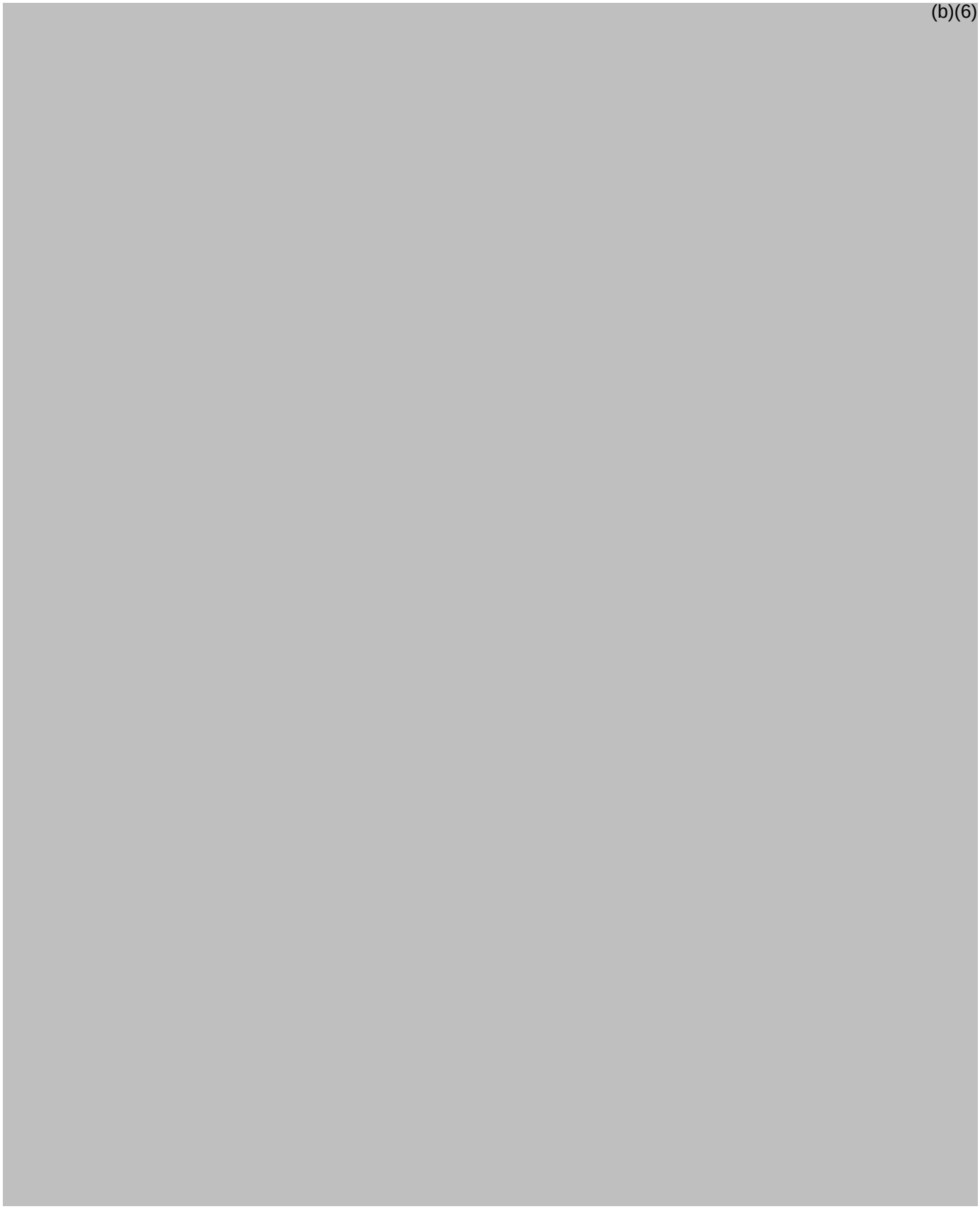
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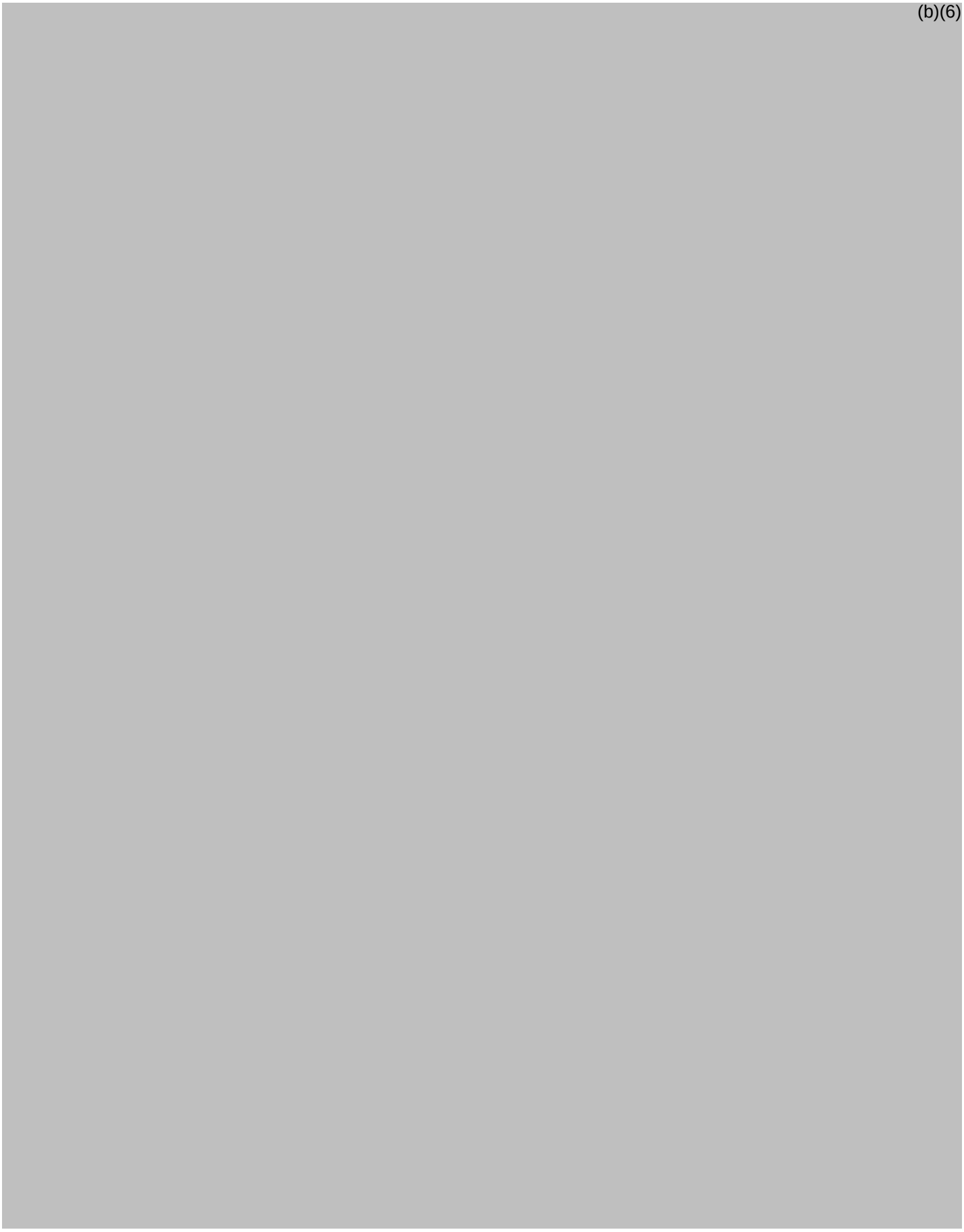

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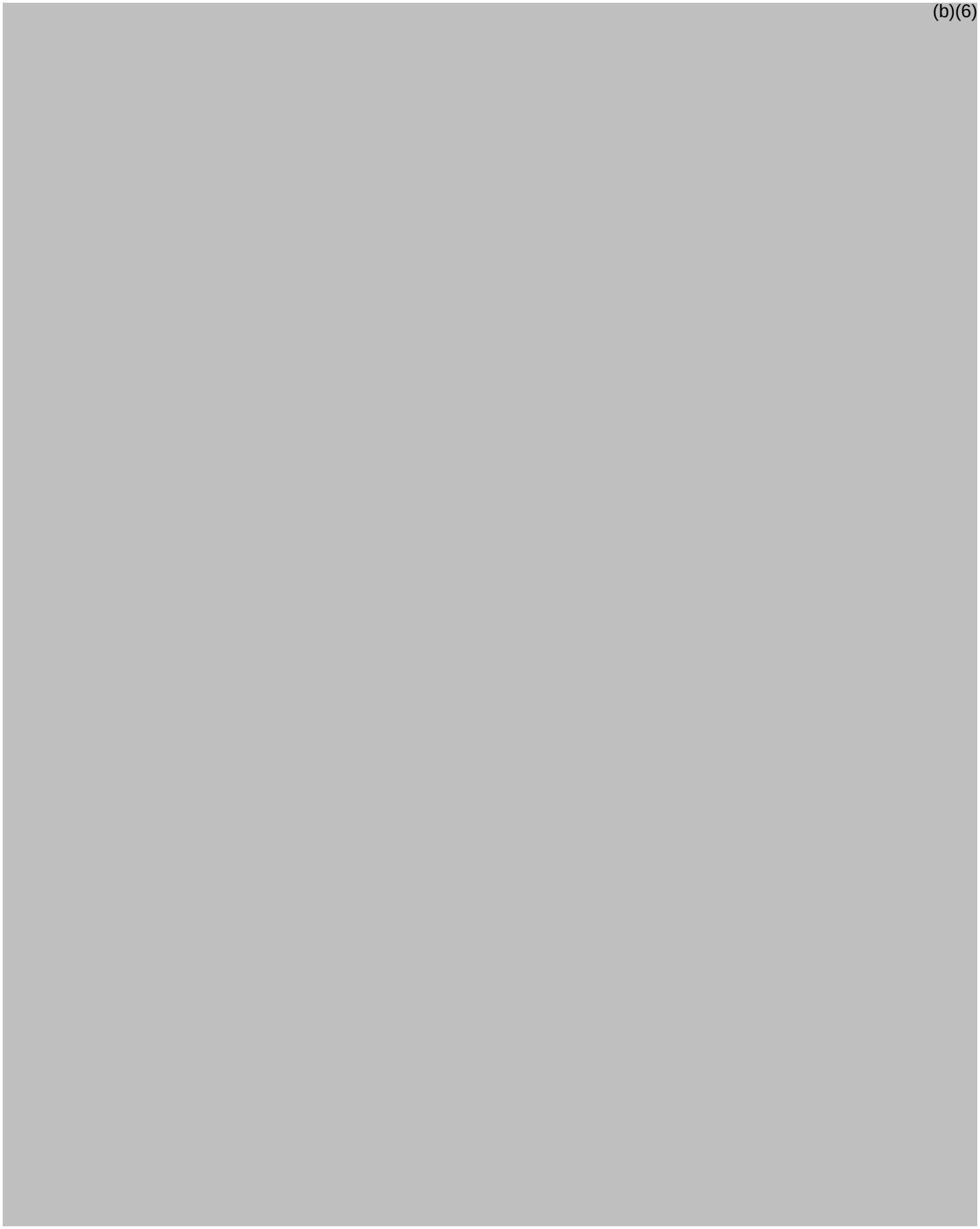

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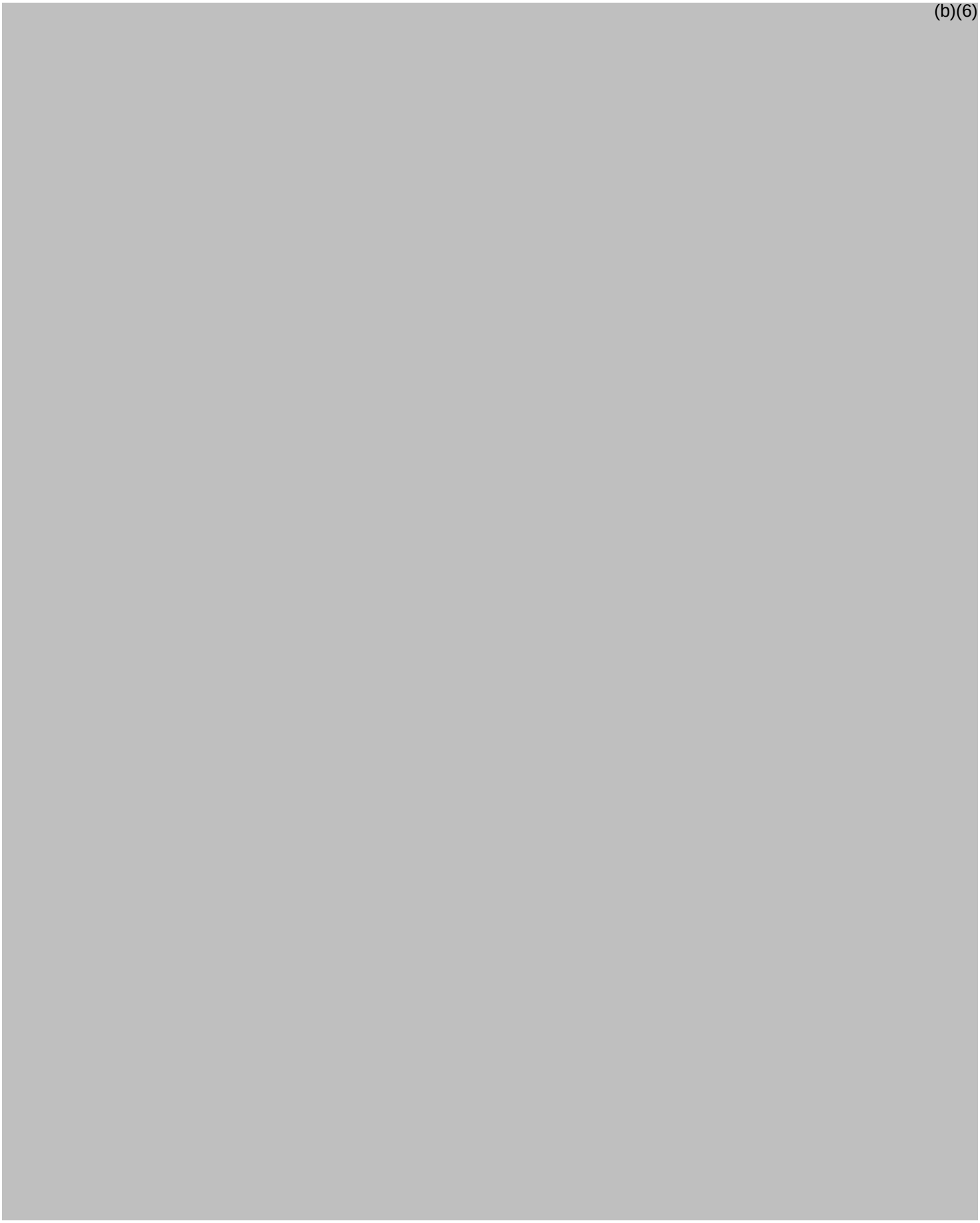

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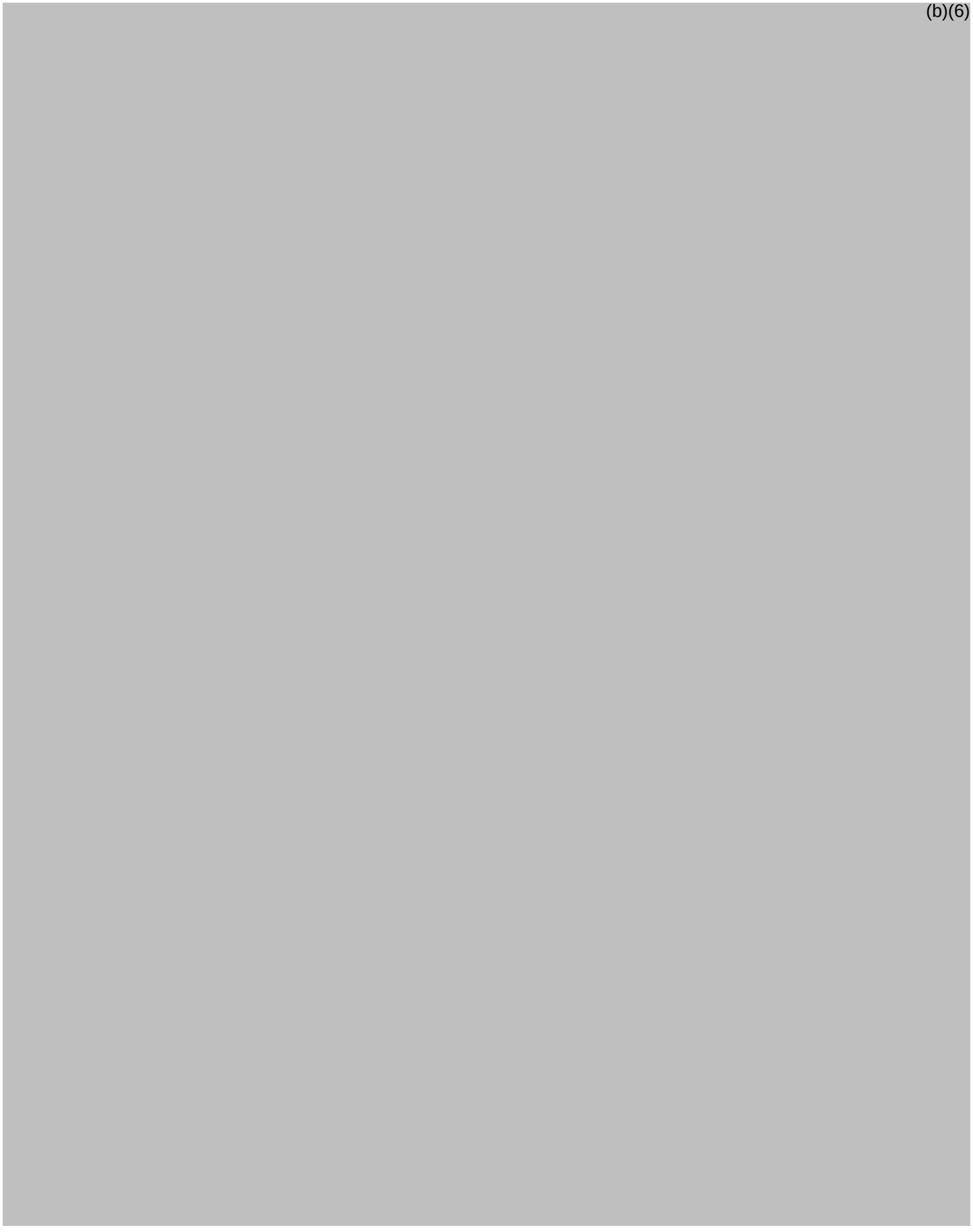

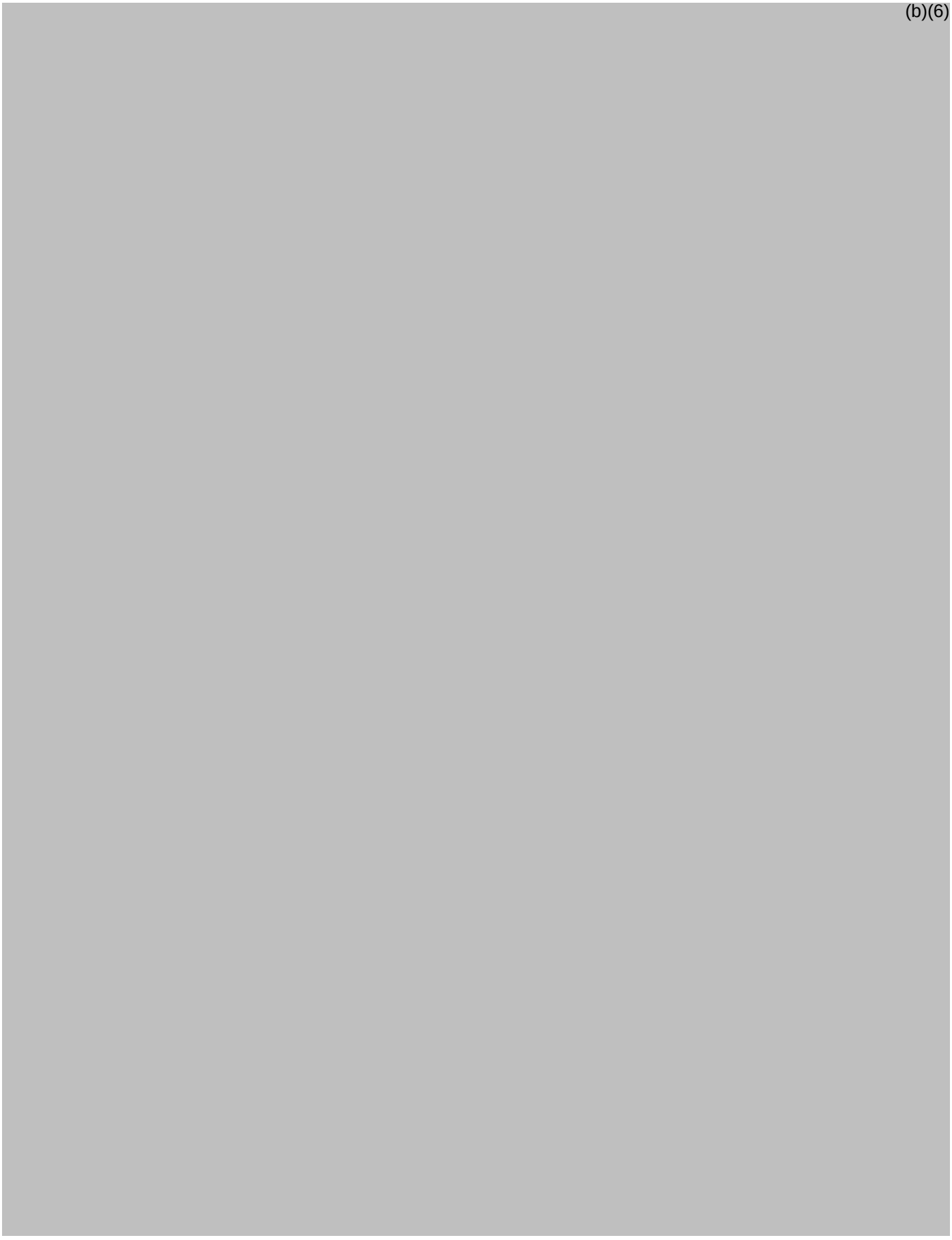
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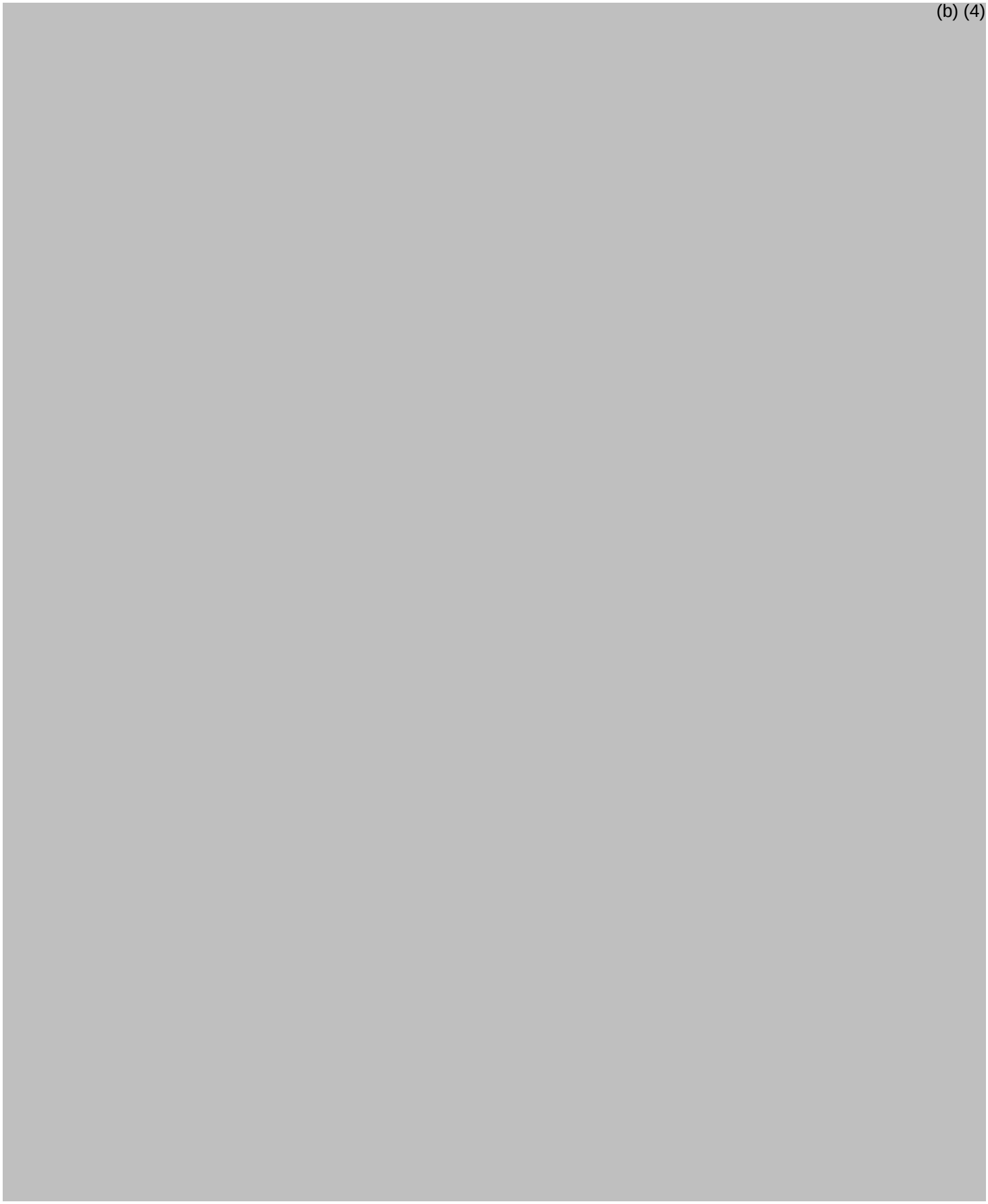

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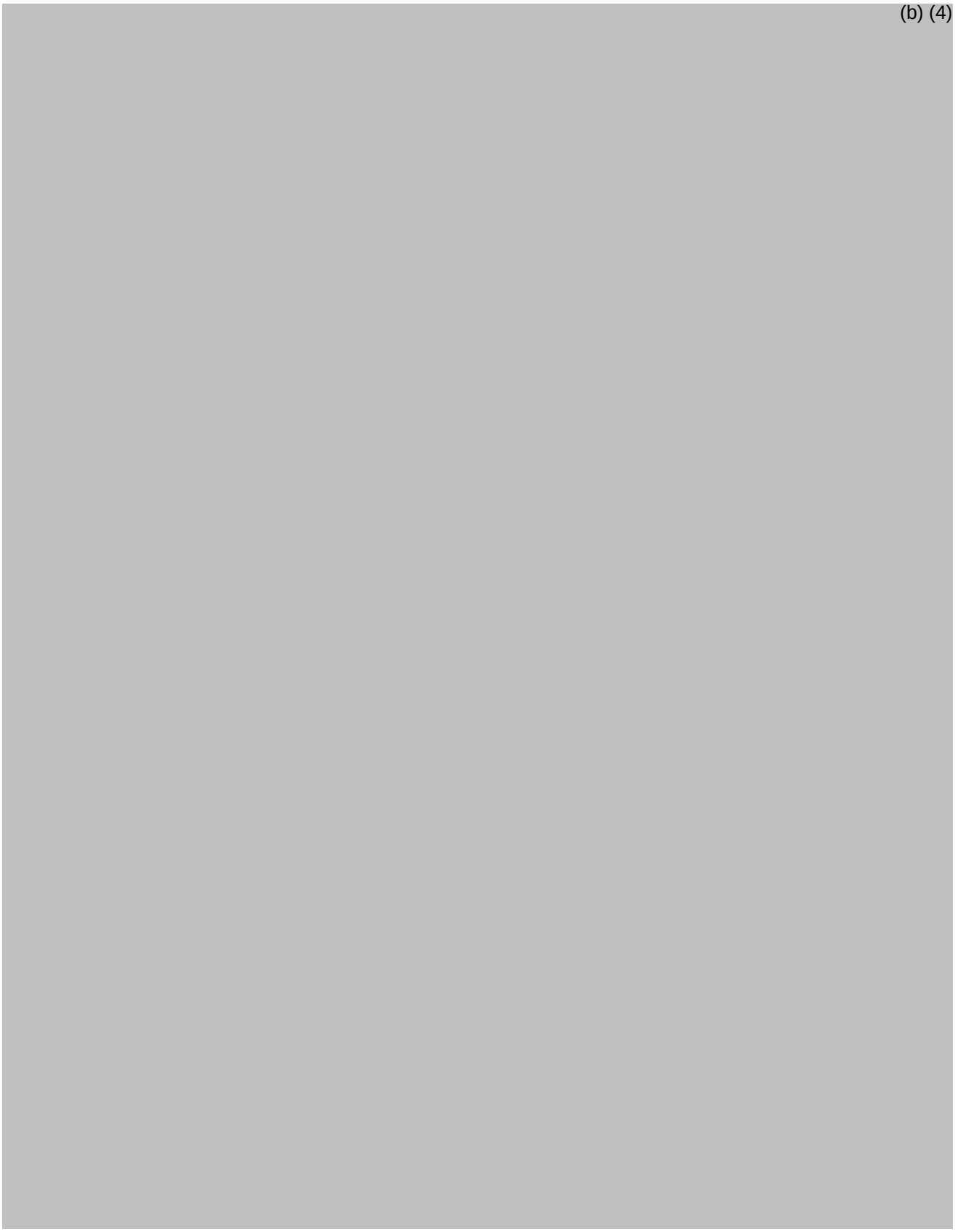

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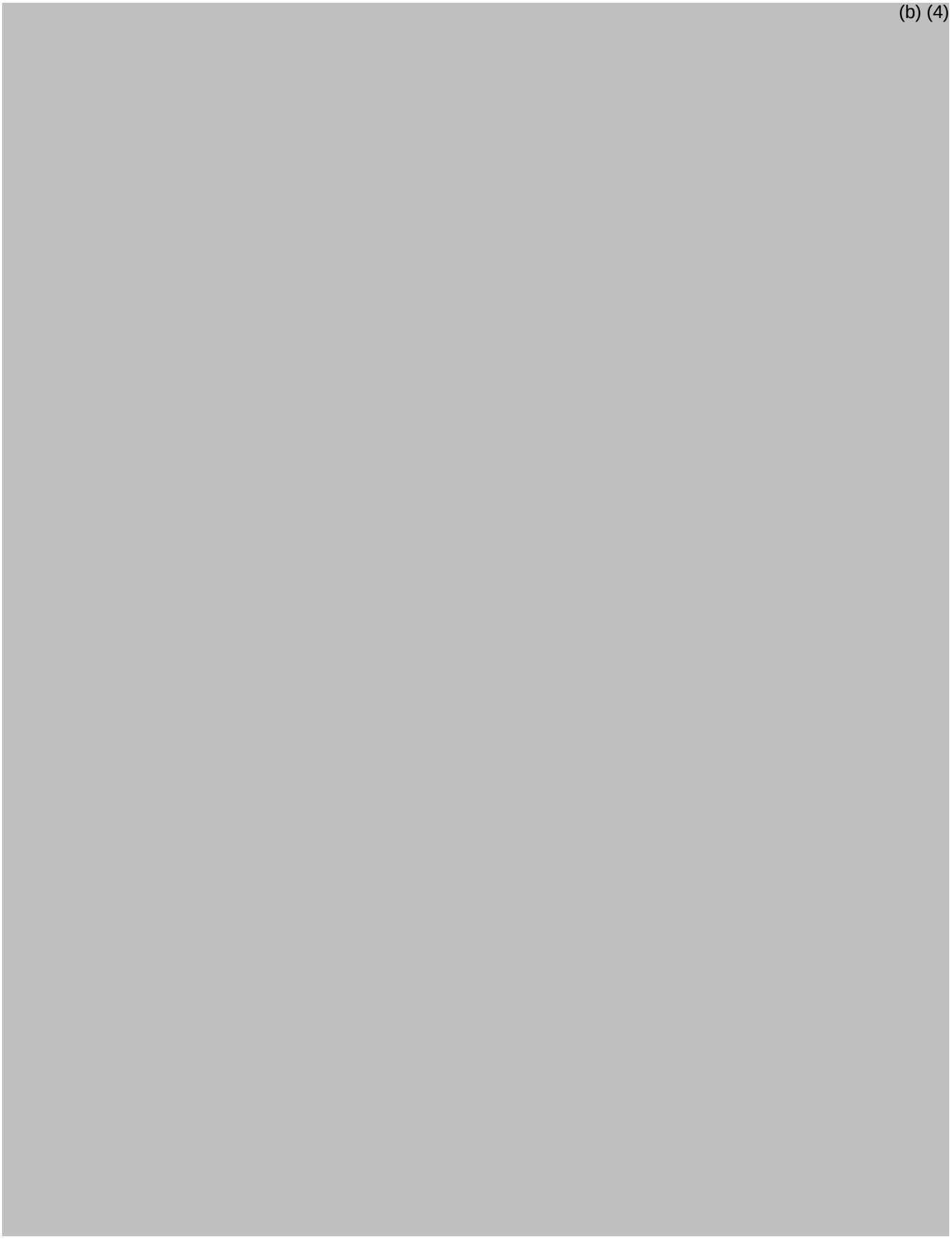

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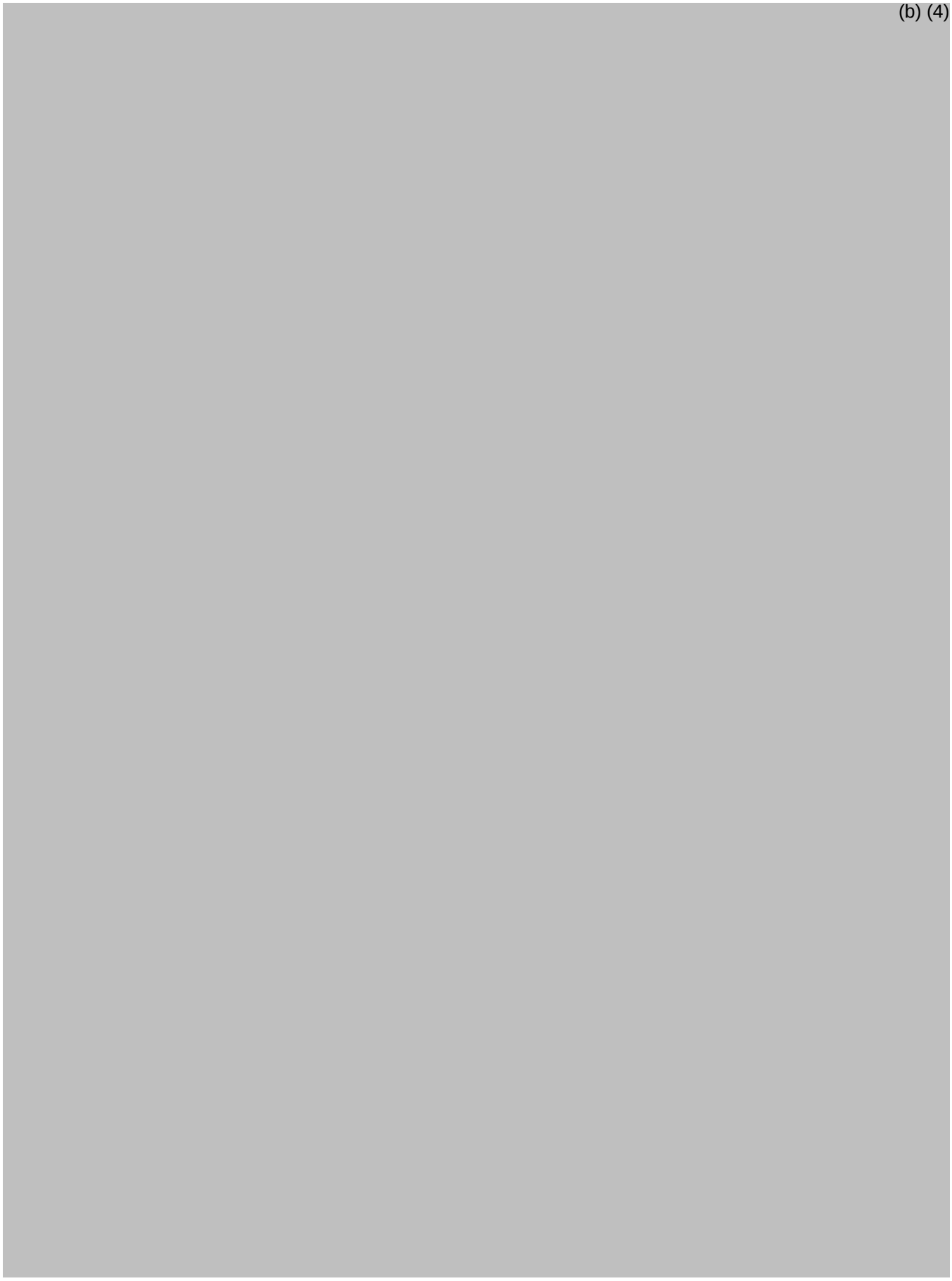

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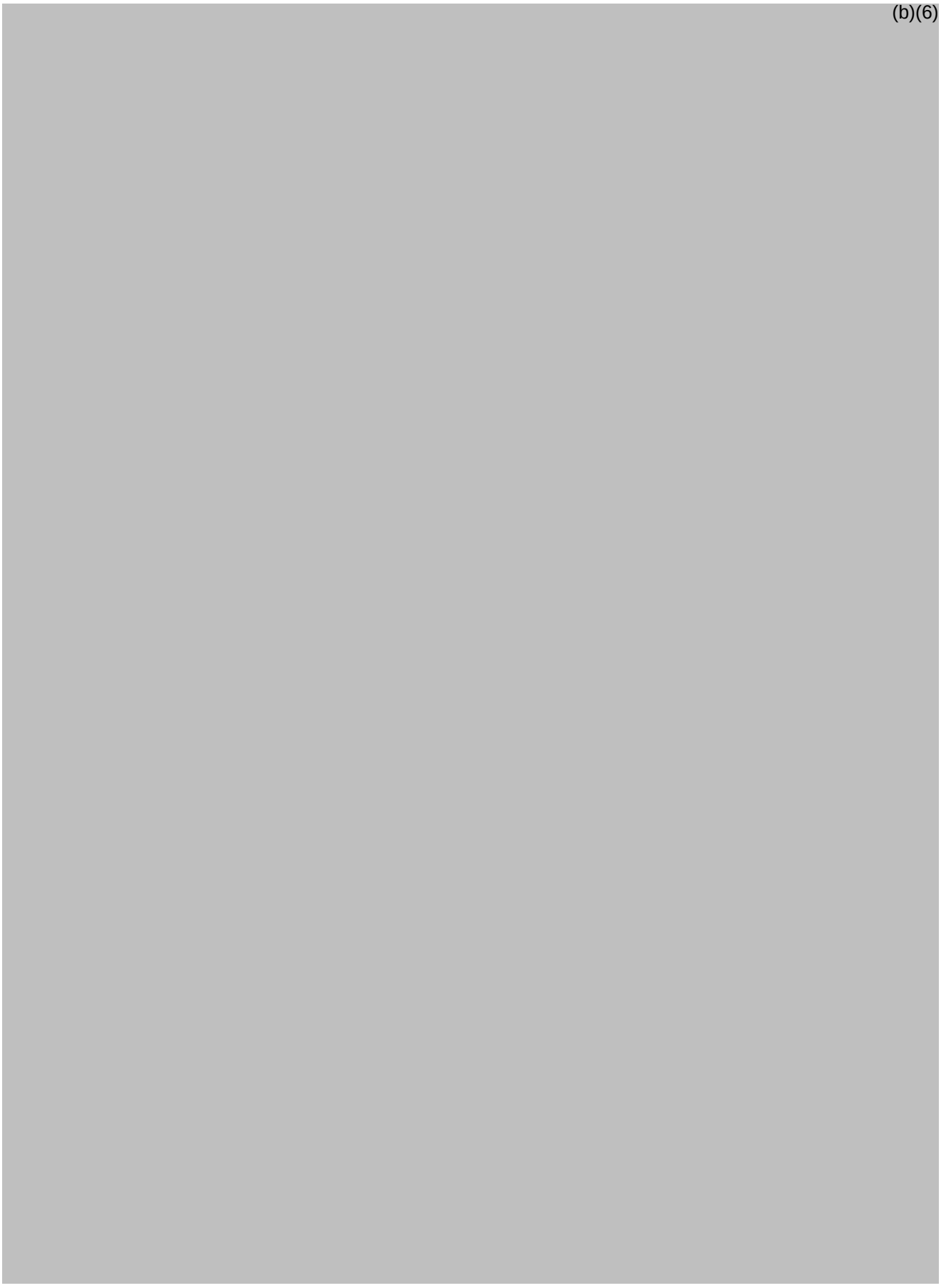

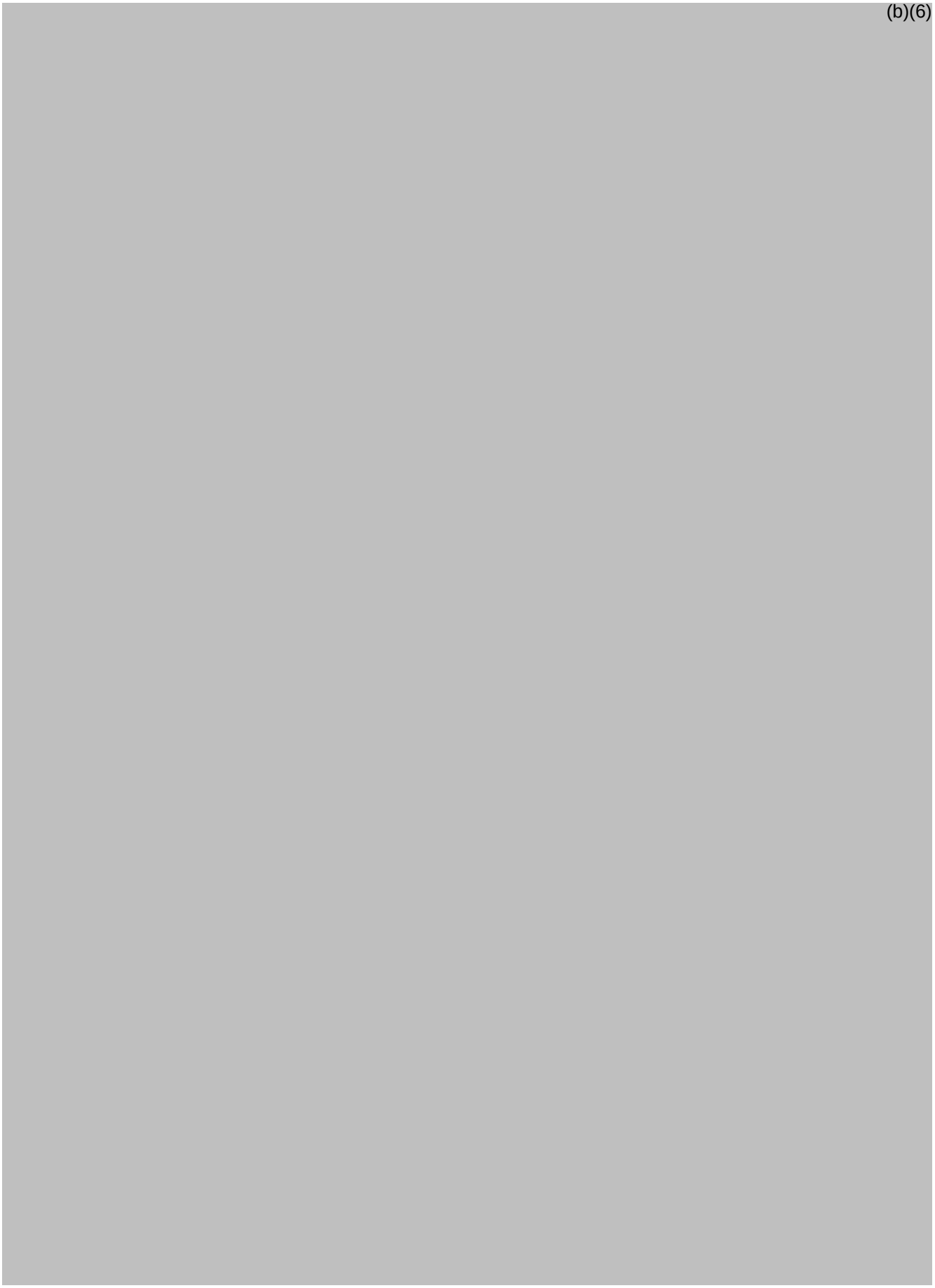
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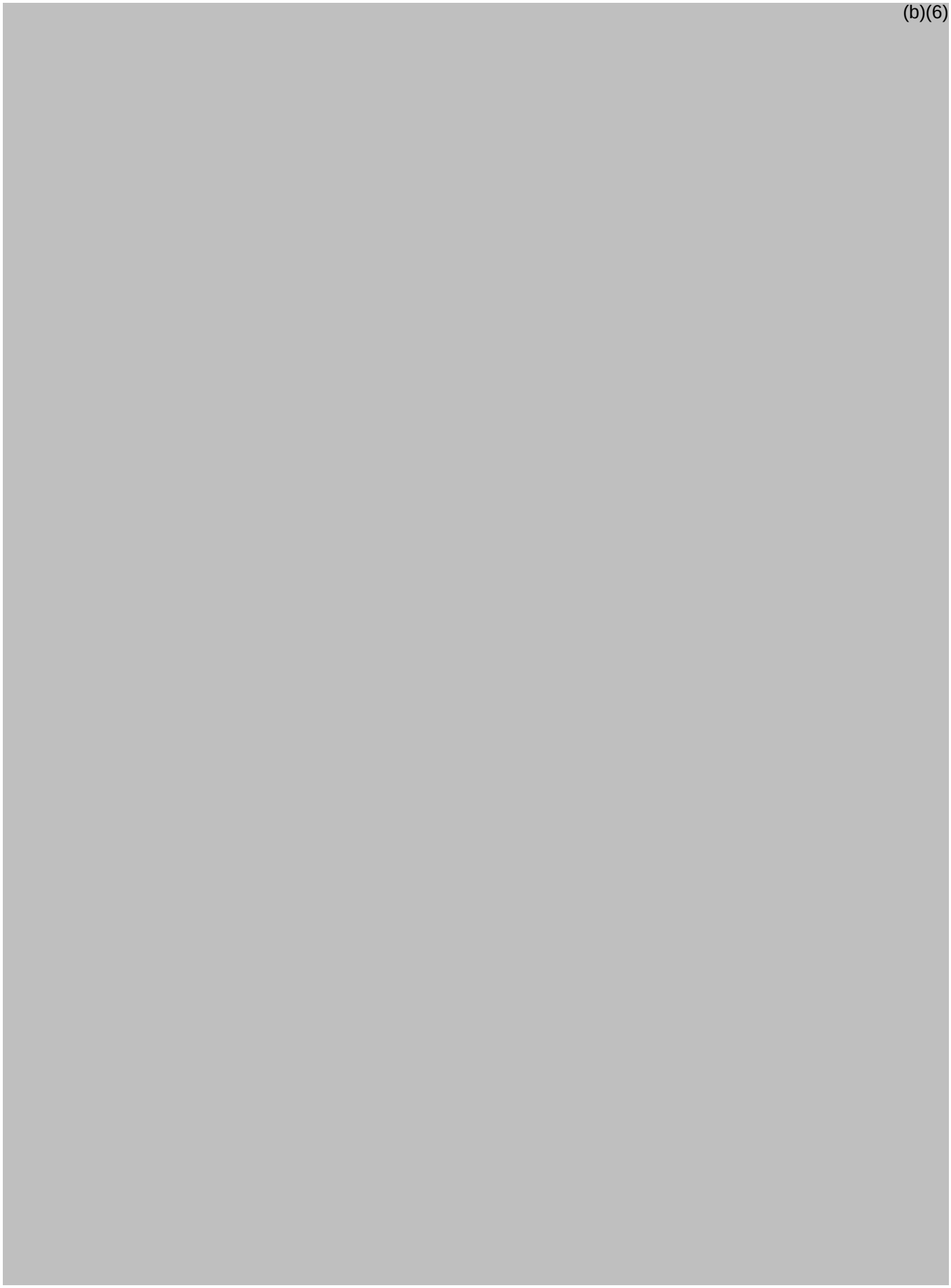
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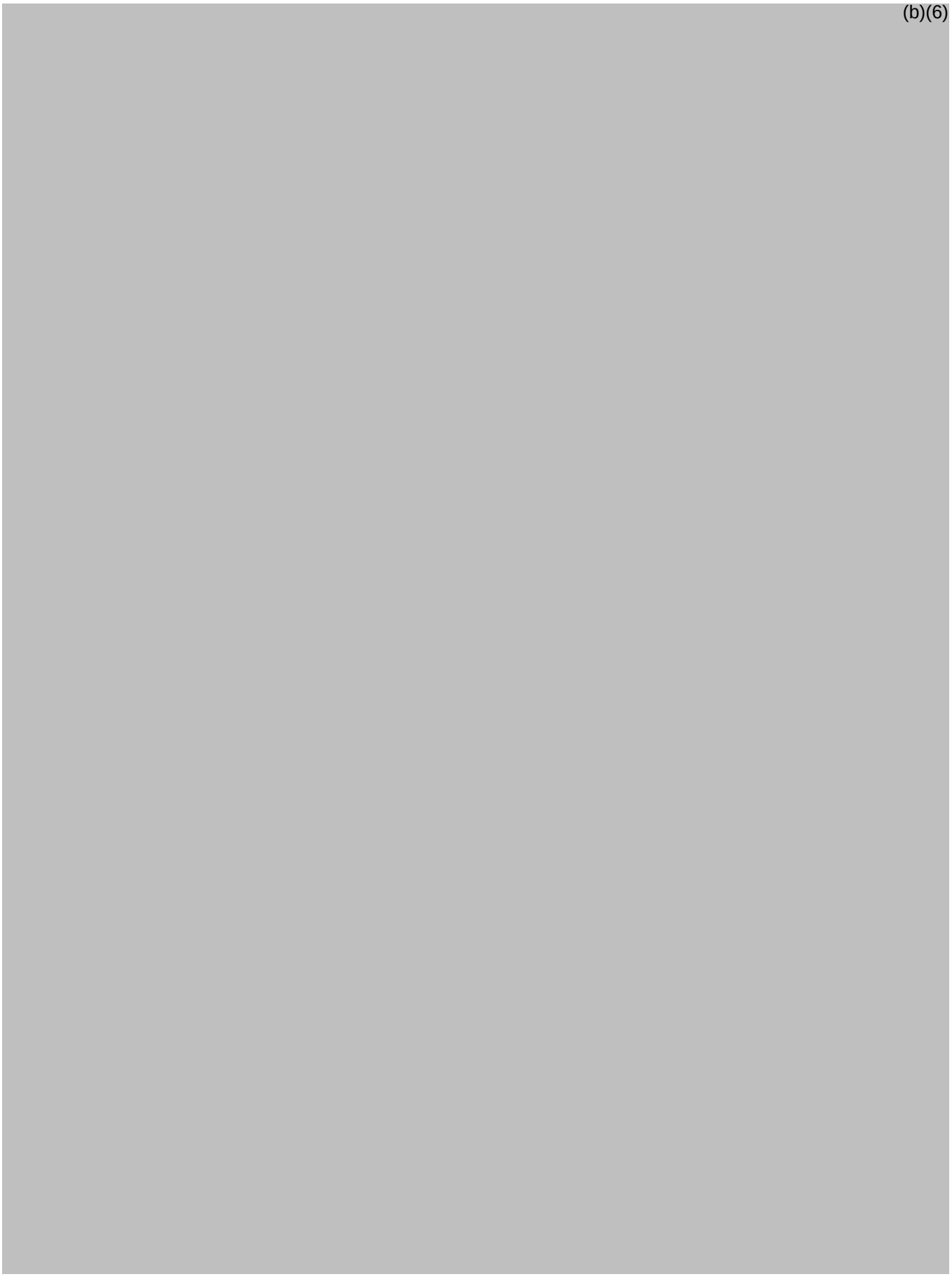

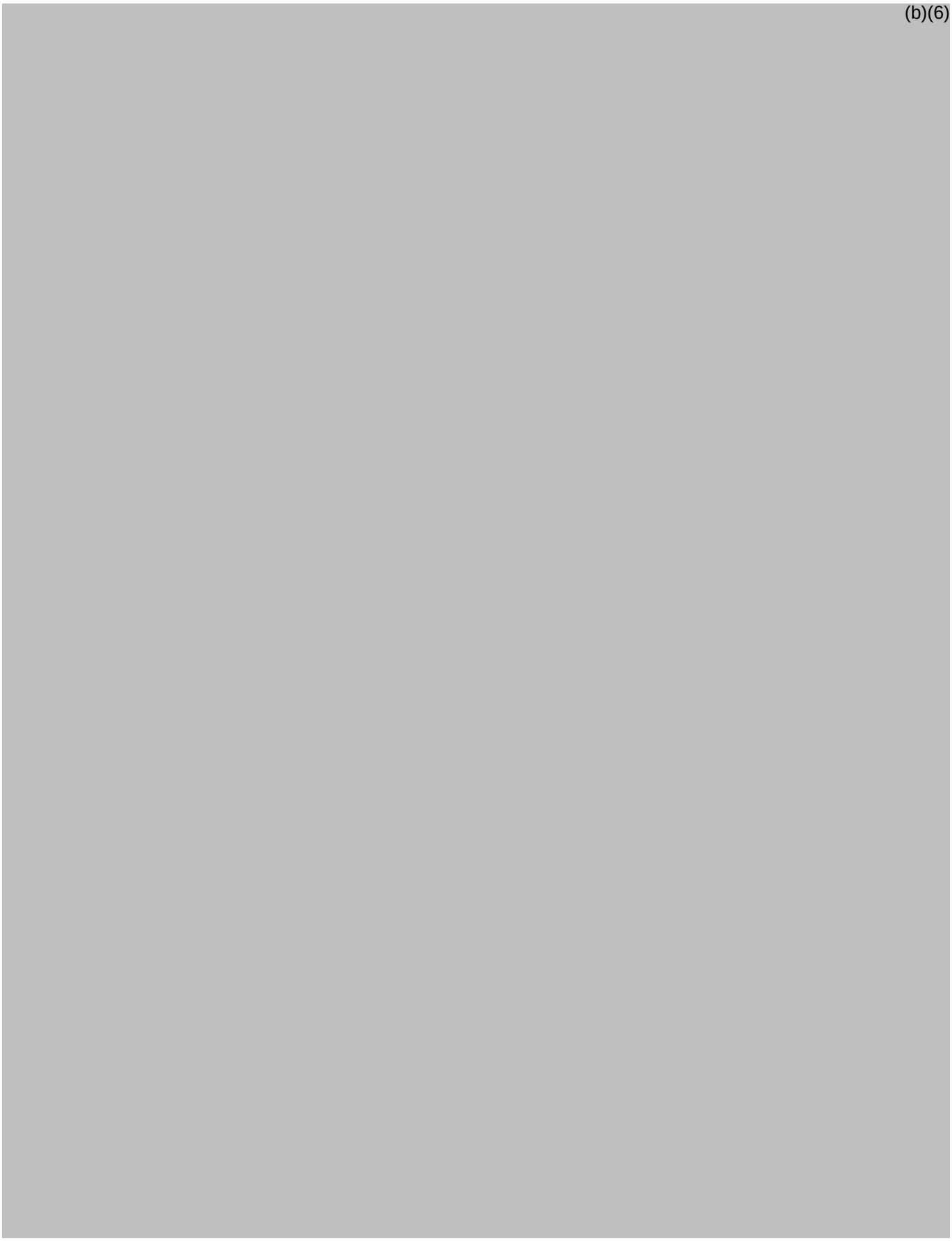
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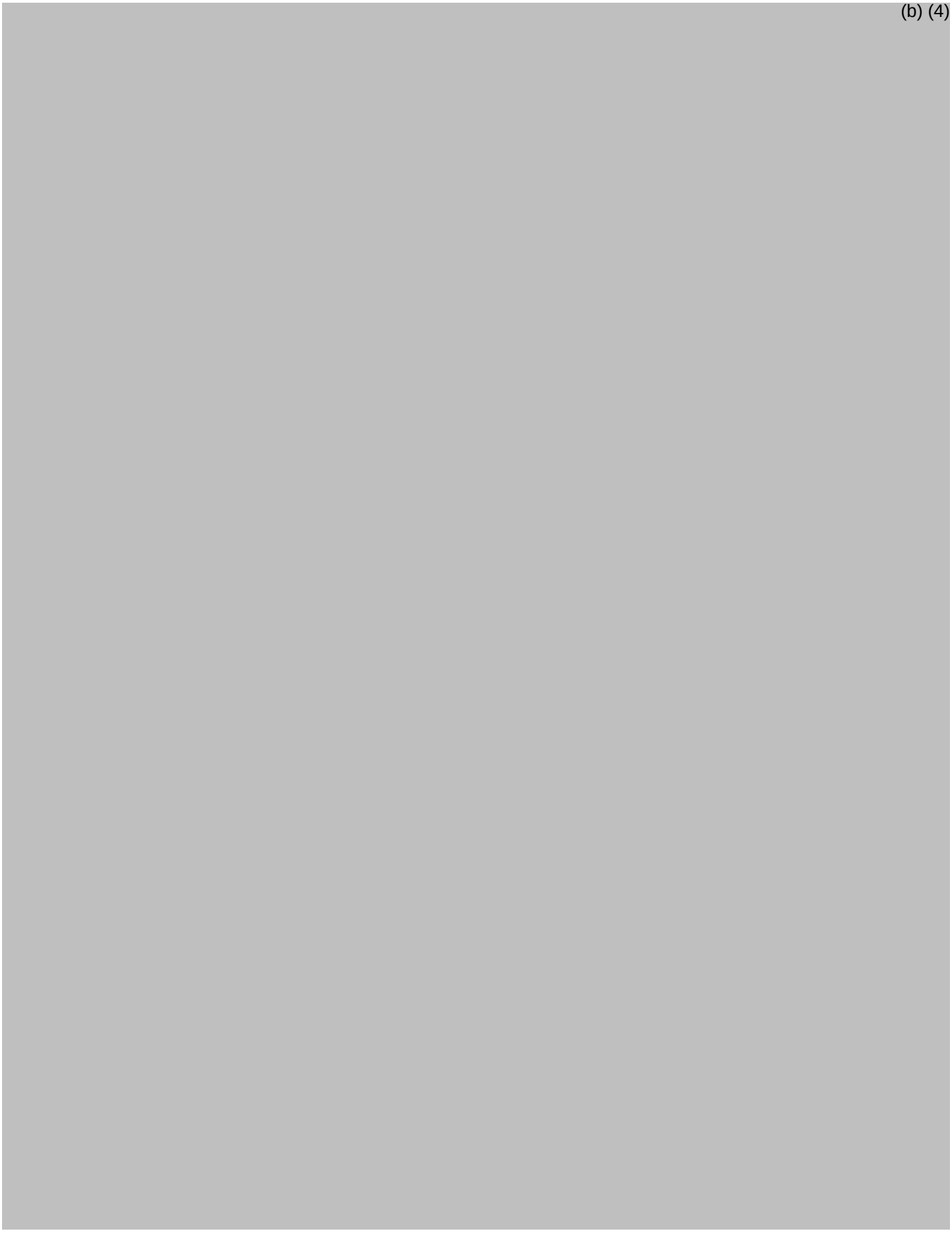

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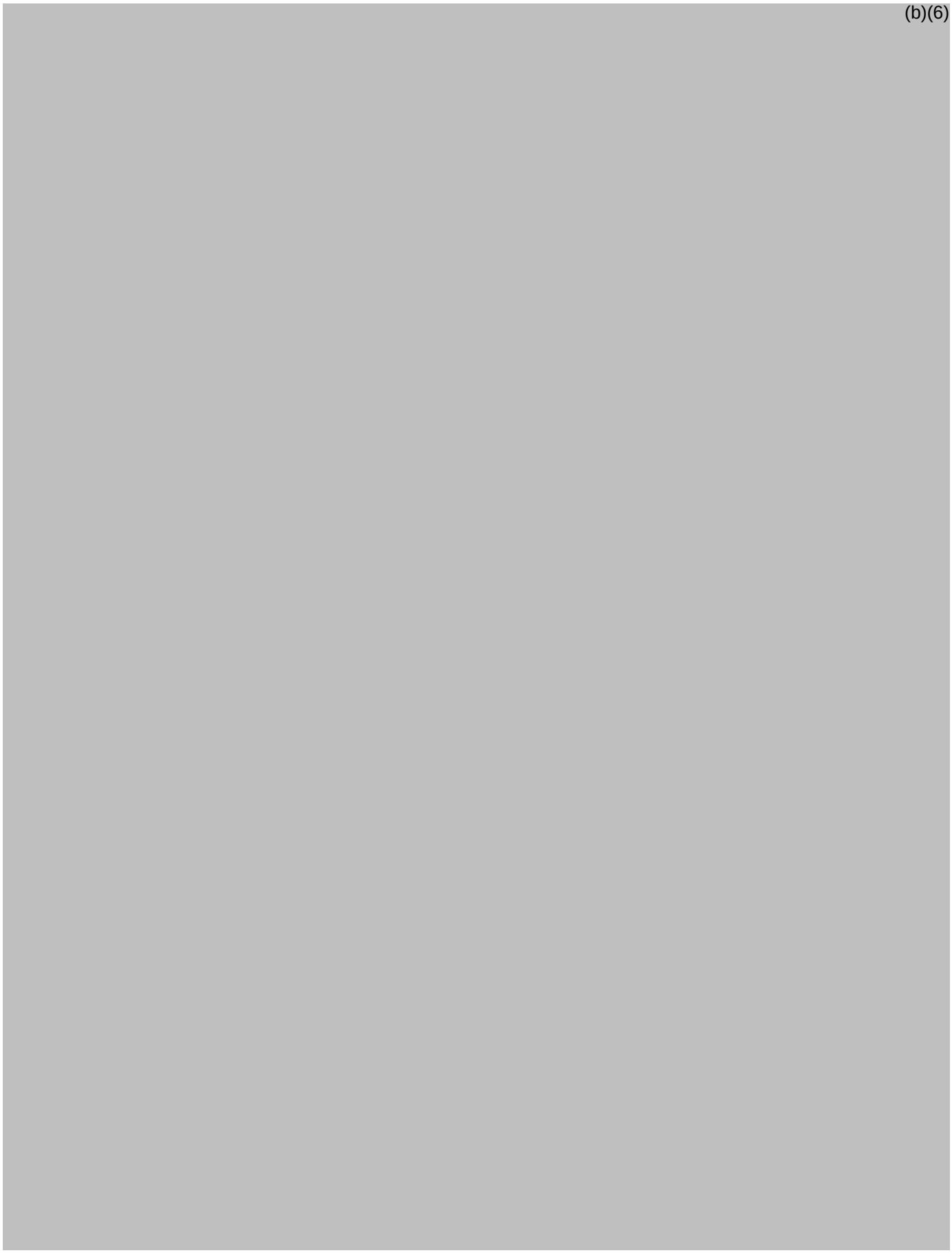

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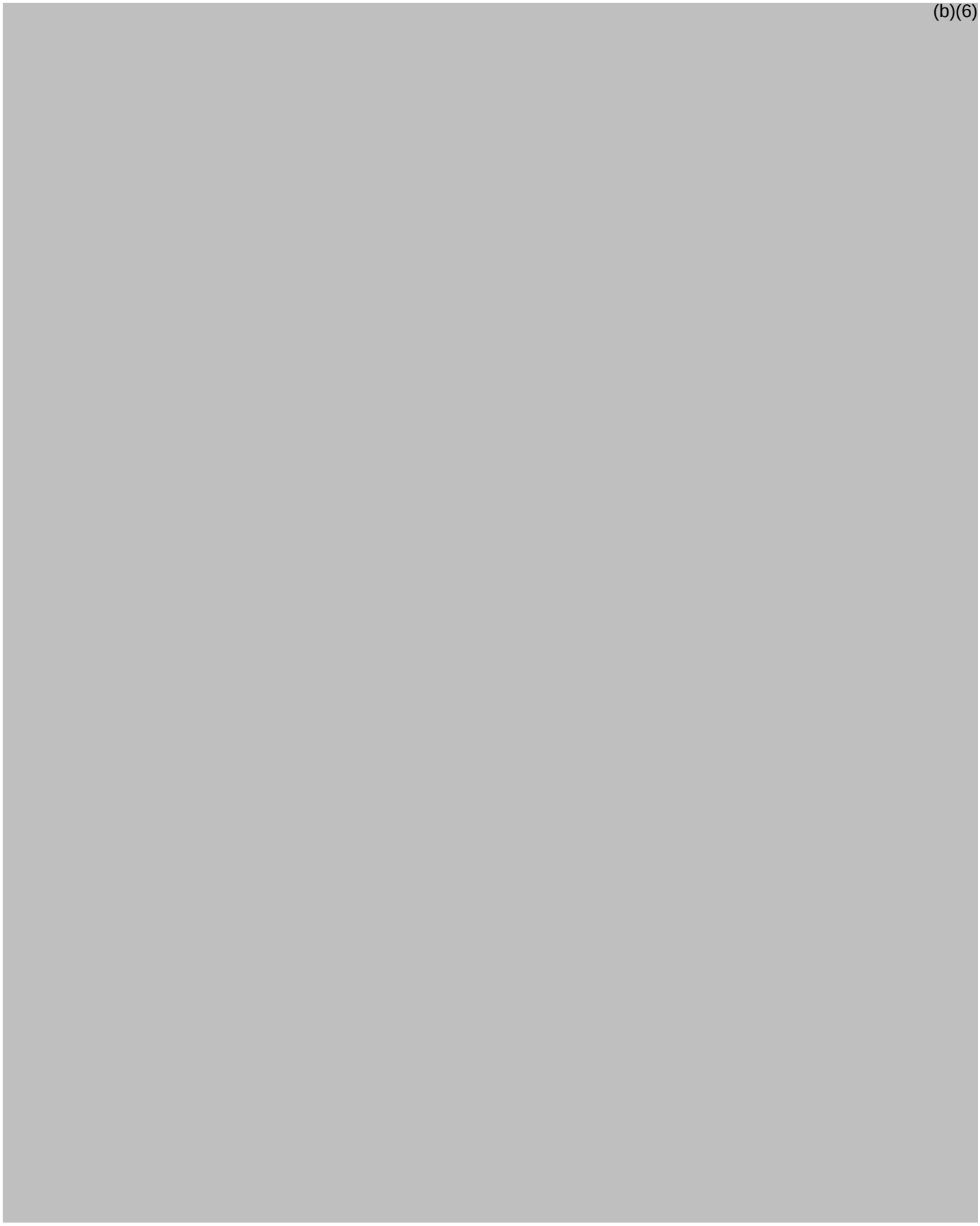

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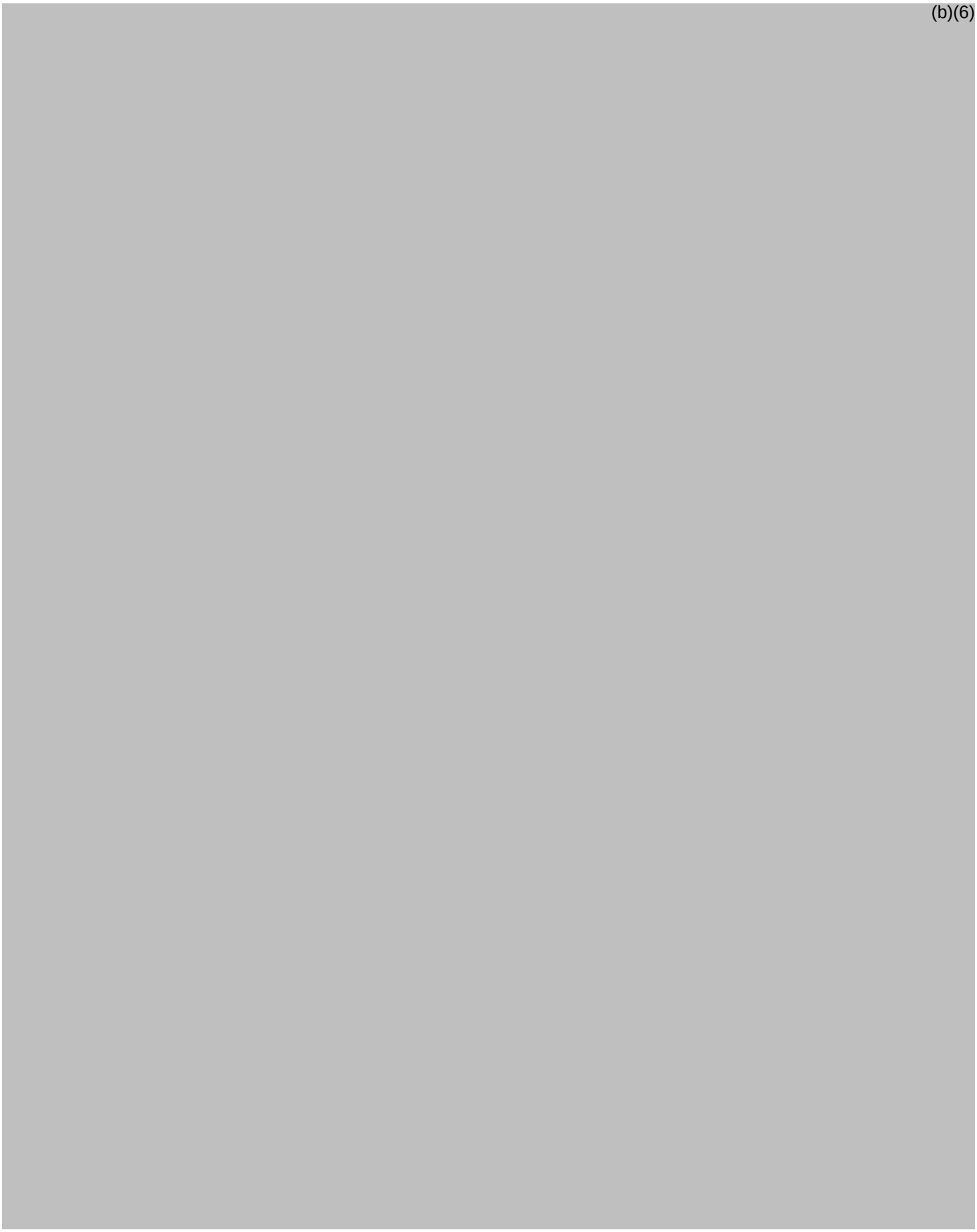


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Curriculum Vitae of

DANIEL WIERDA, M.S., Ph.D., Fellow ATS

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SUMMARY OF PROFESSIONAL EXPERIENCE

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EXPERIENCE

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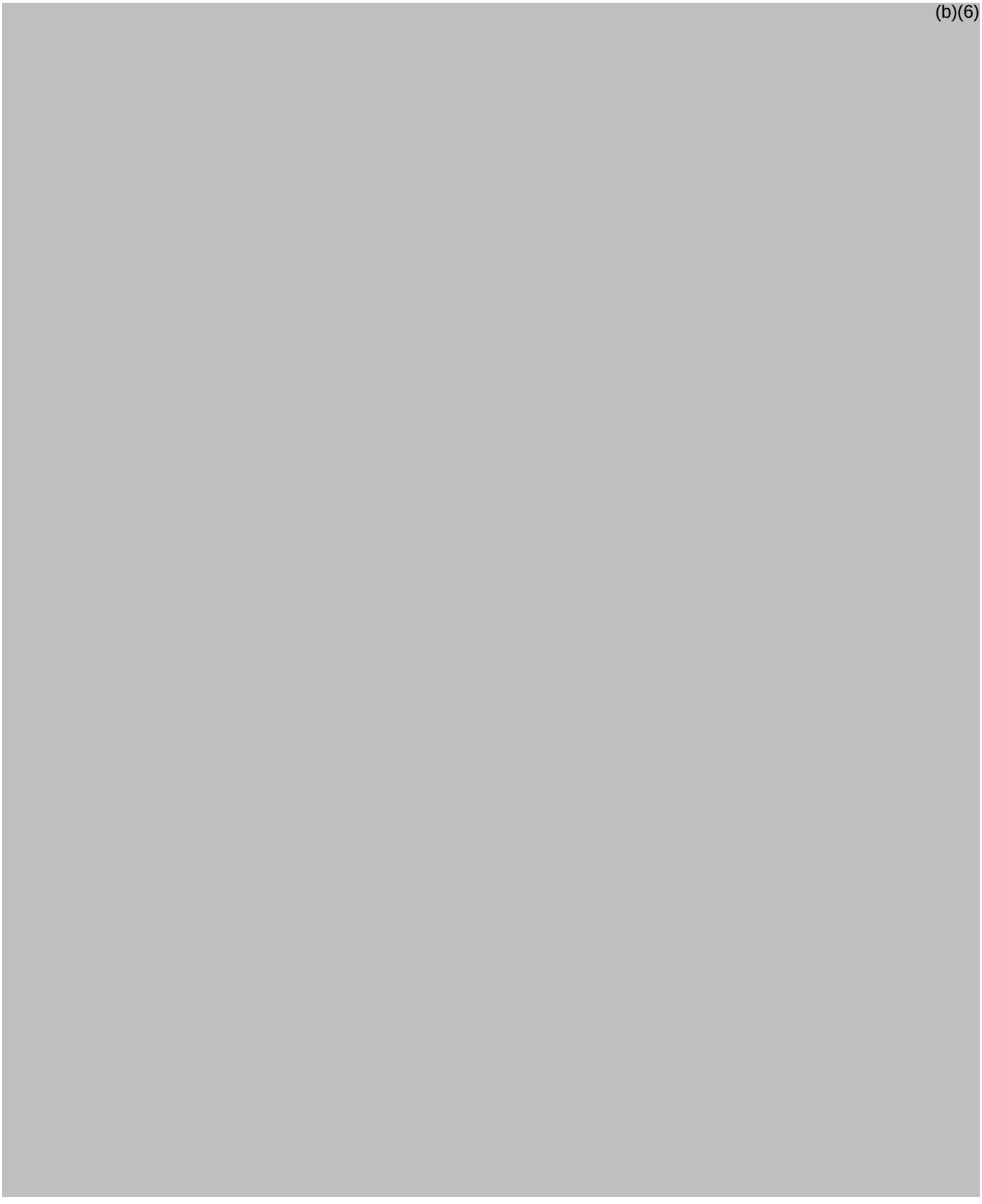

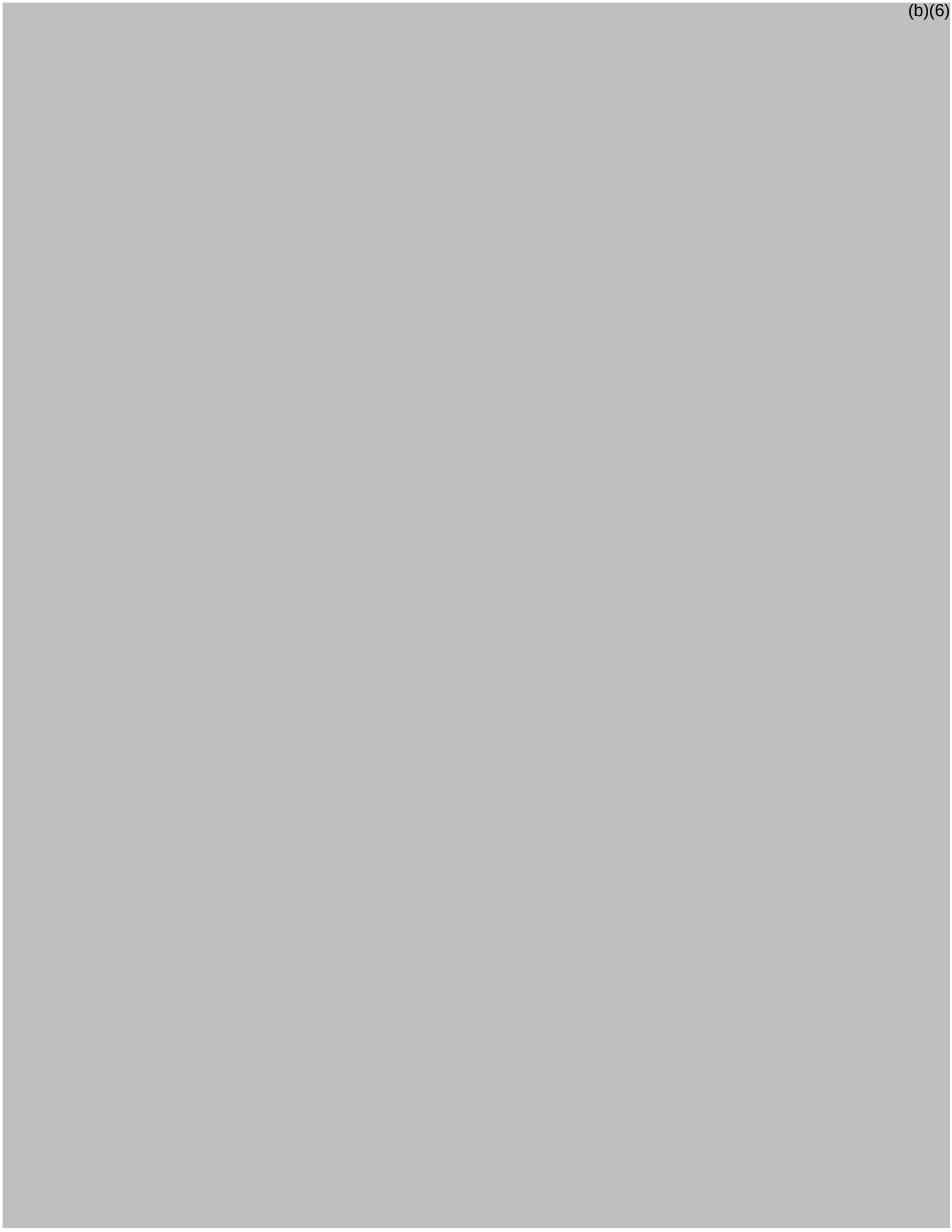
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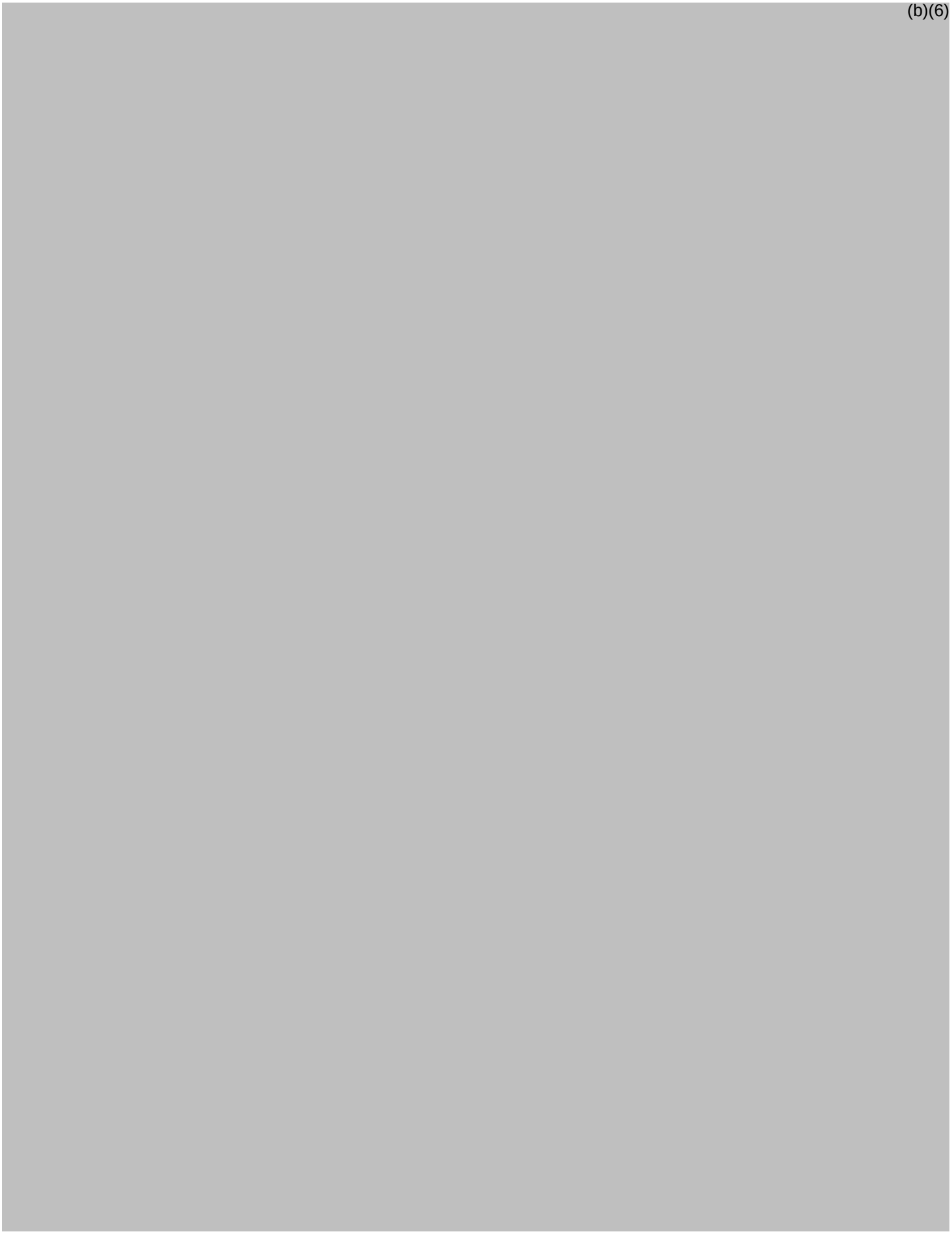
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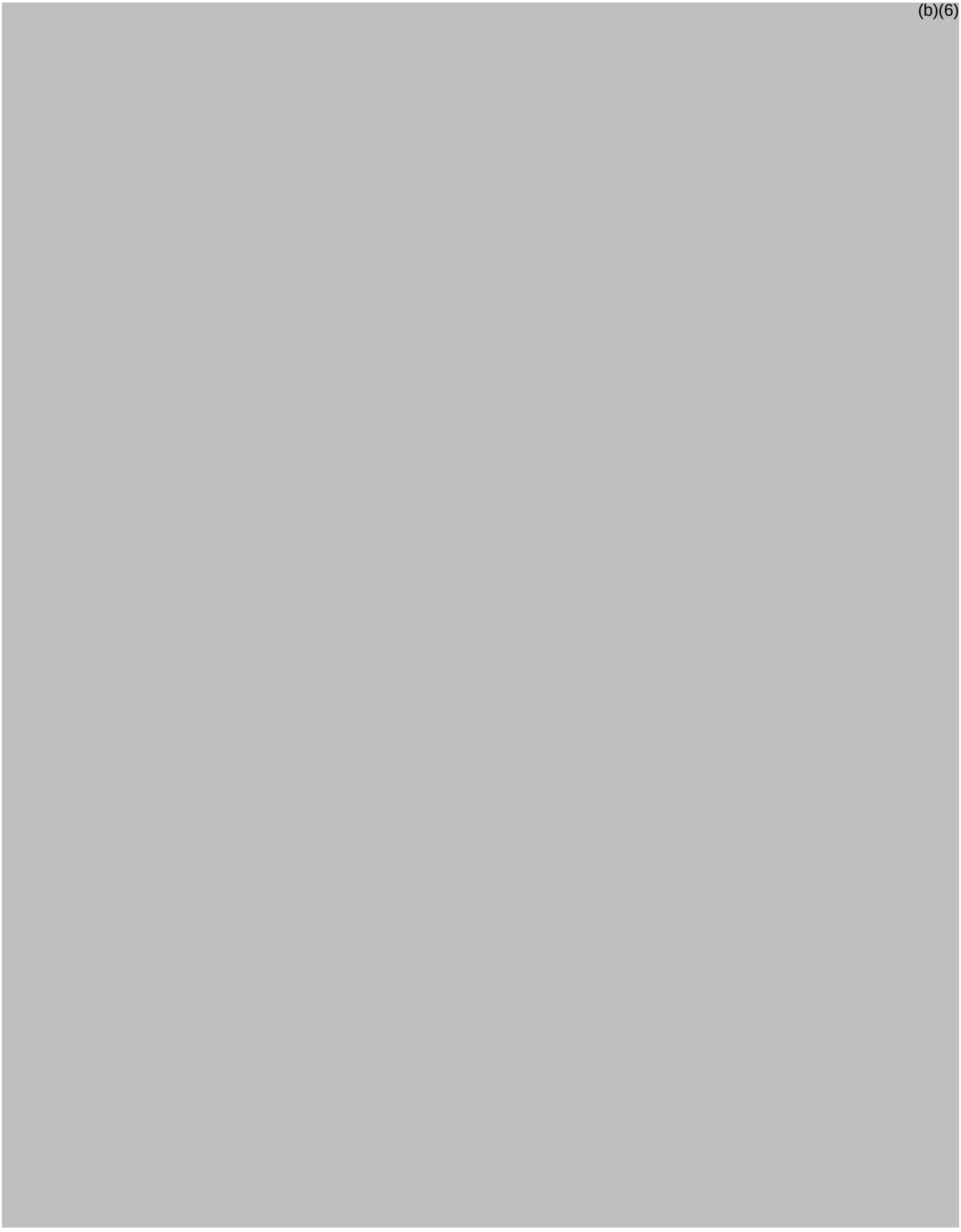
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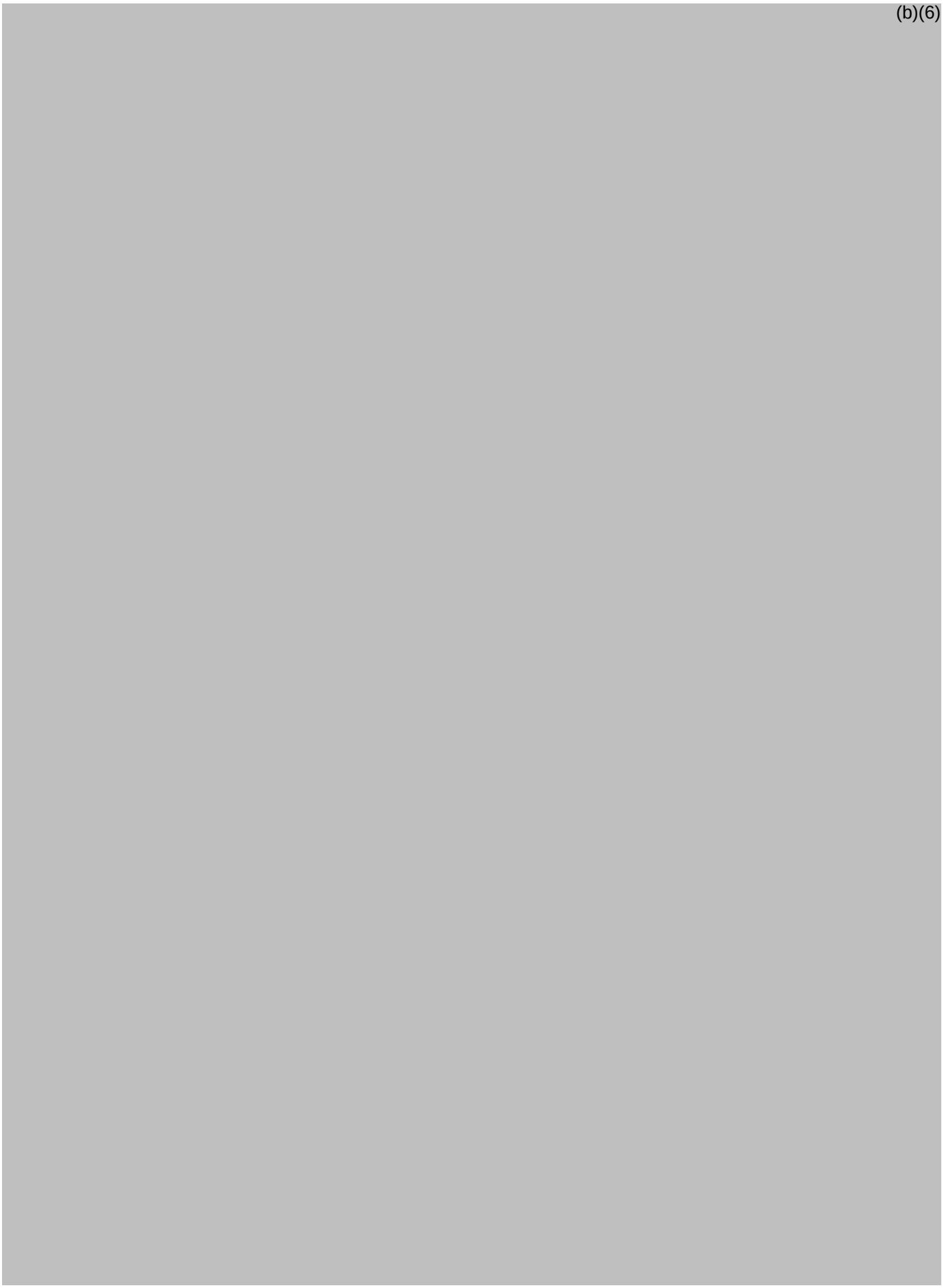
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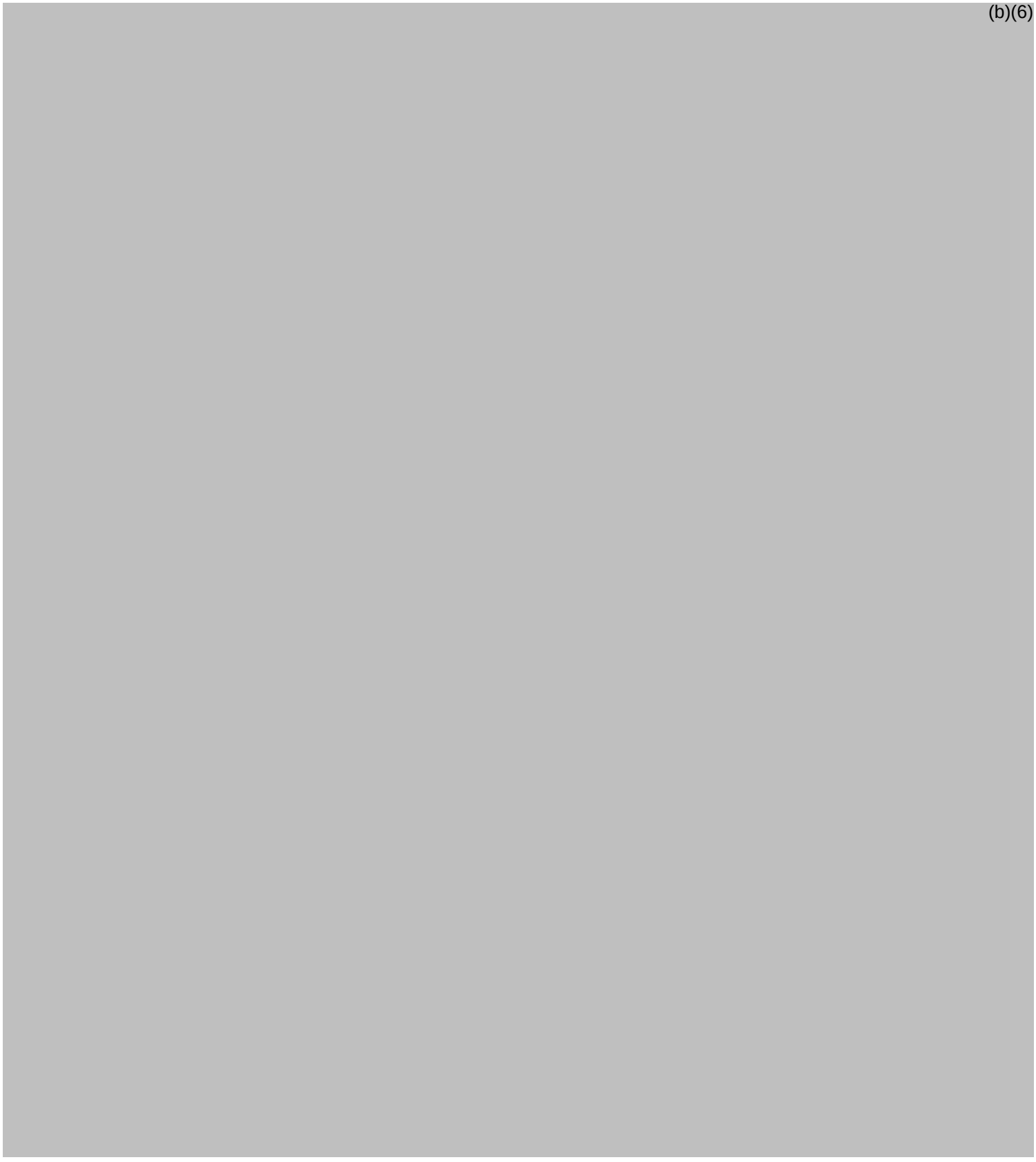
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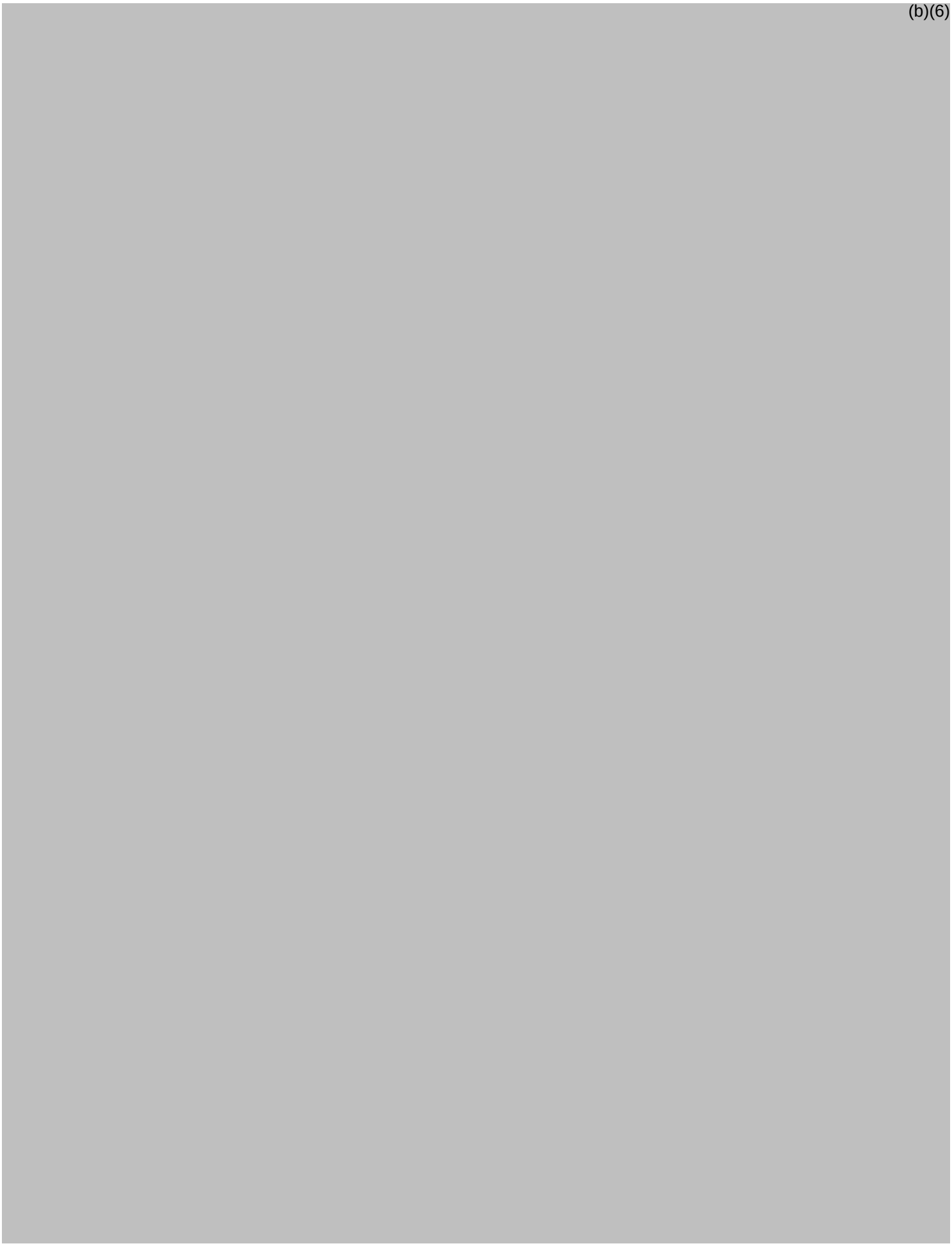
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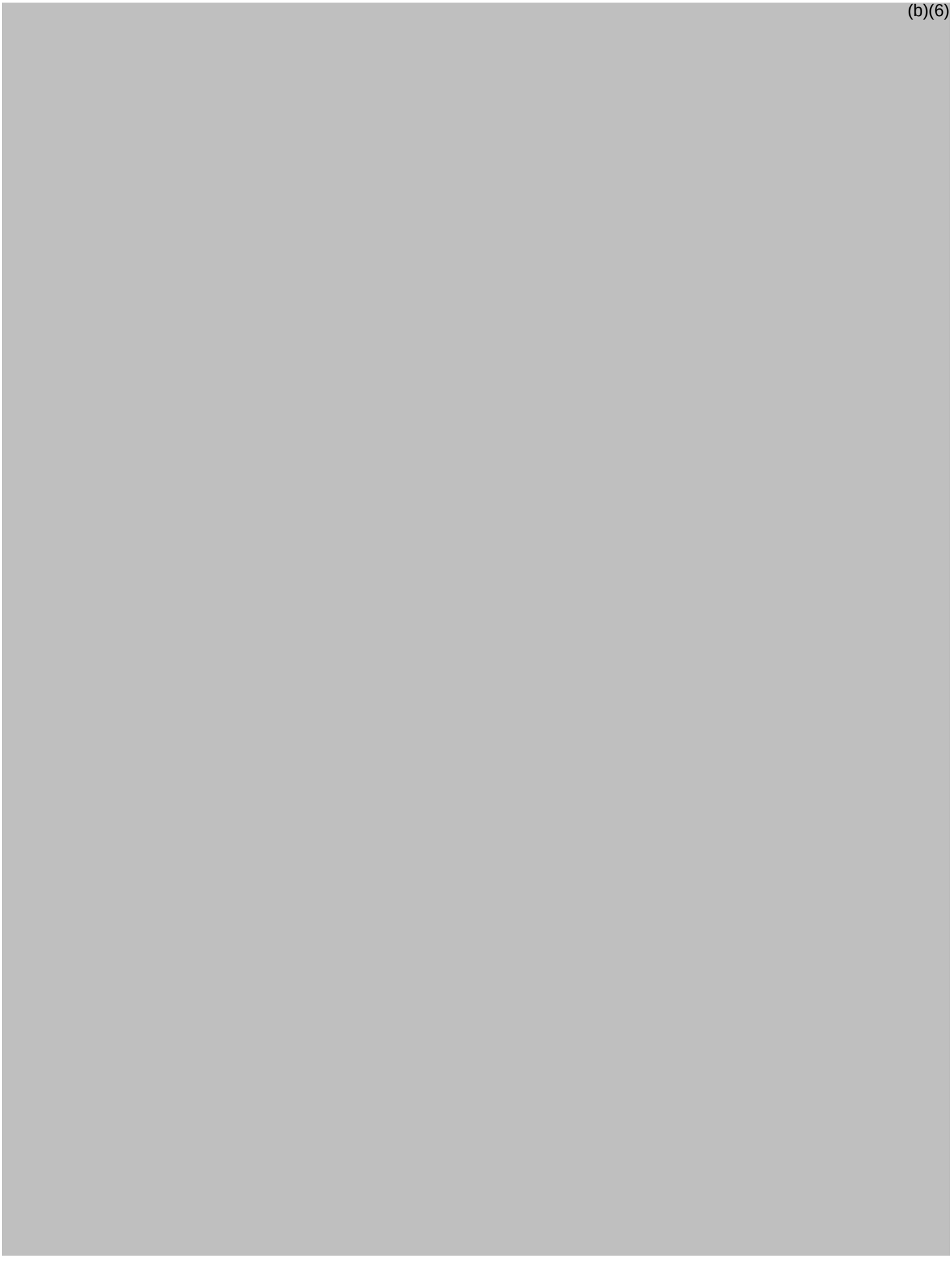

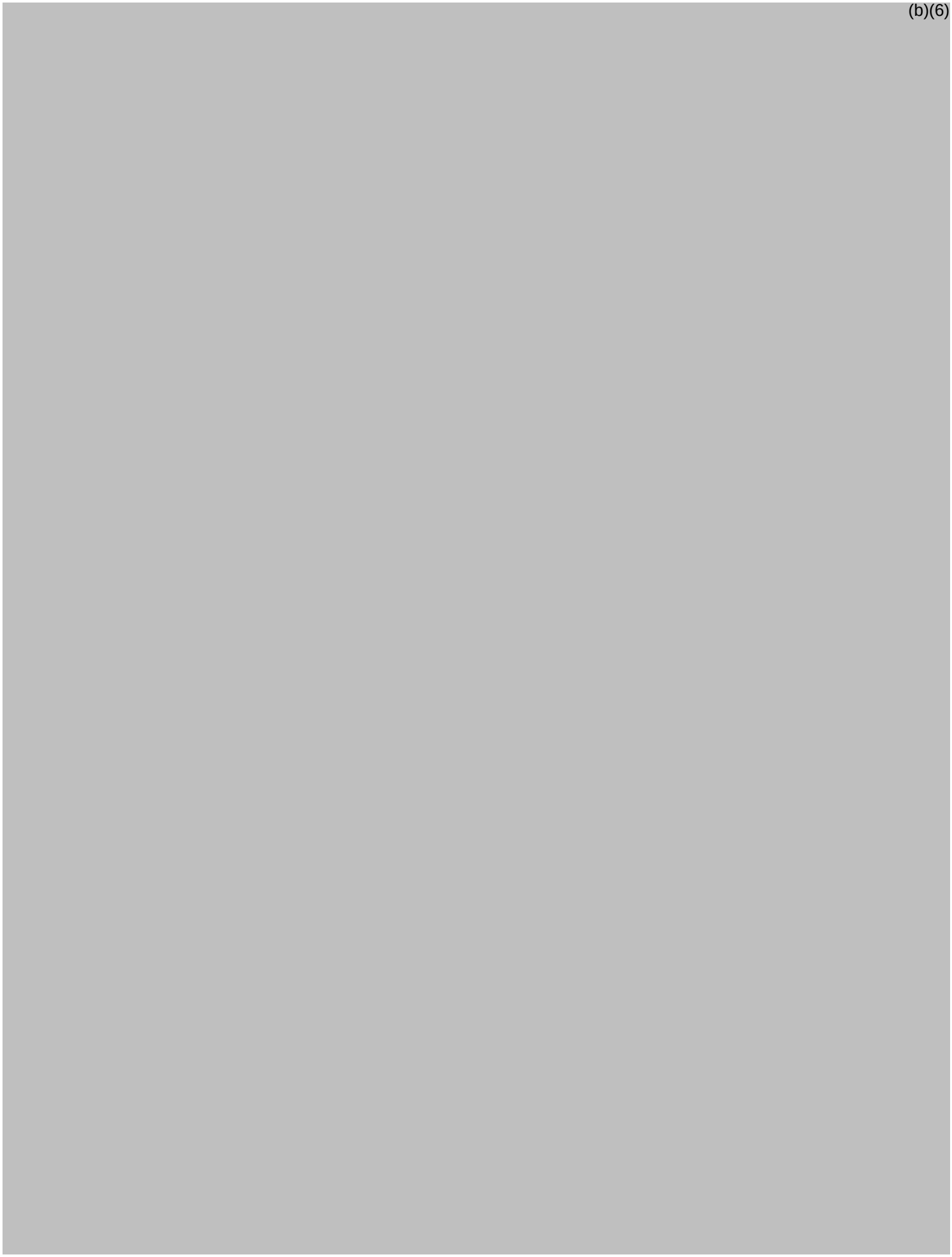

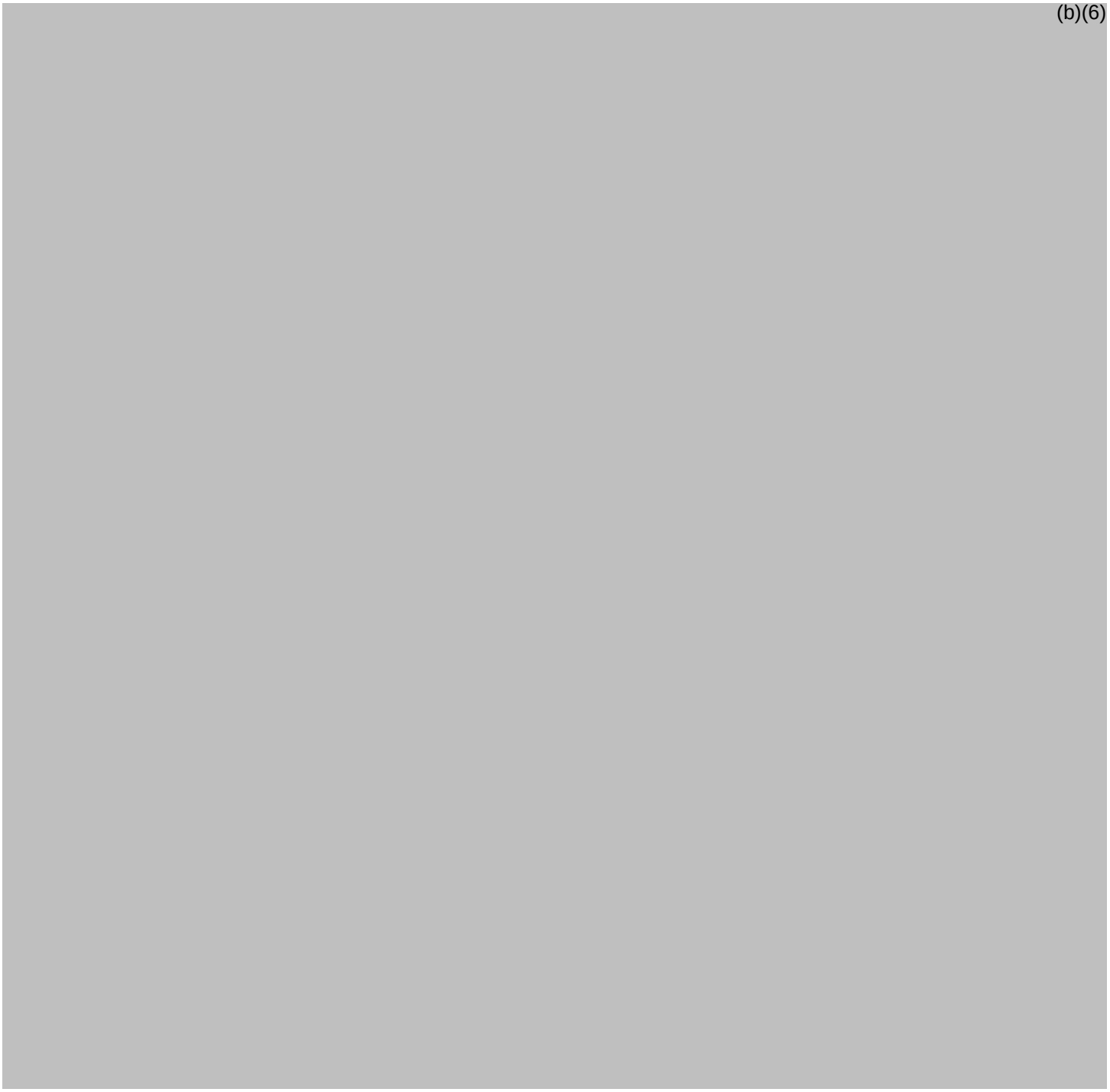
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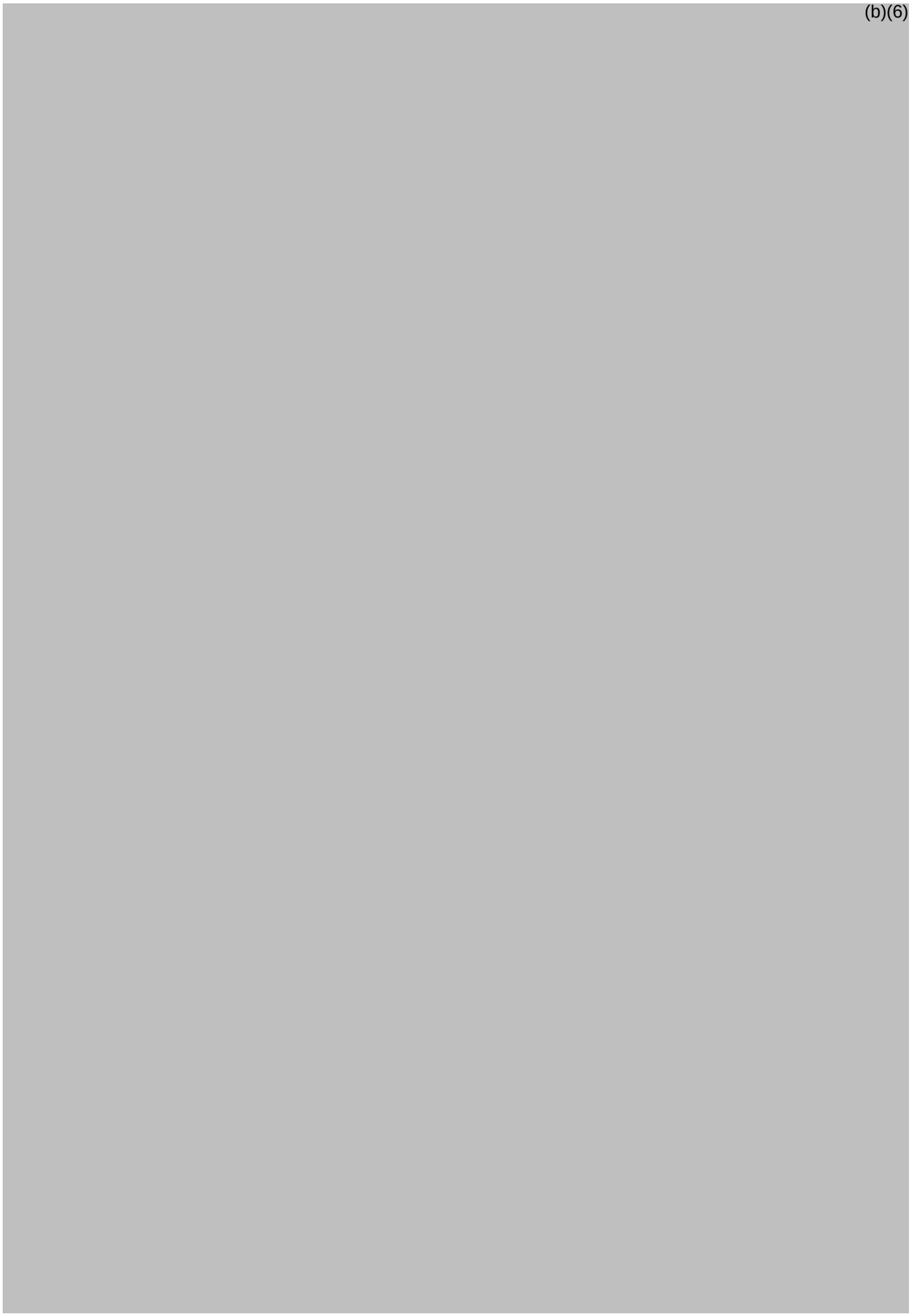
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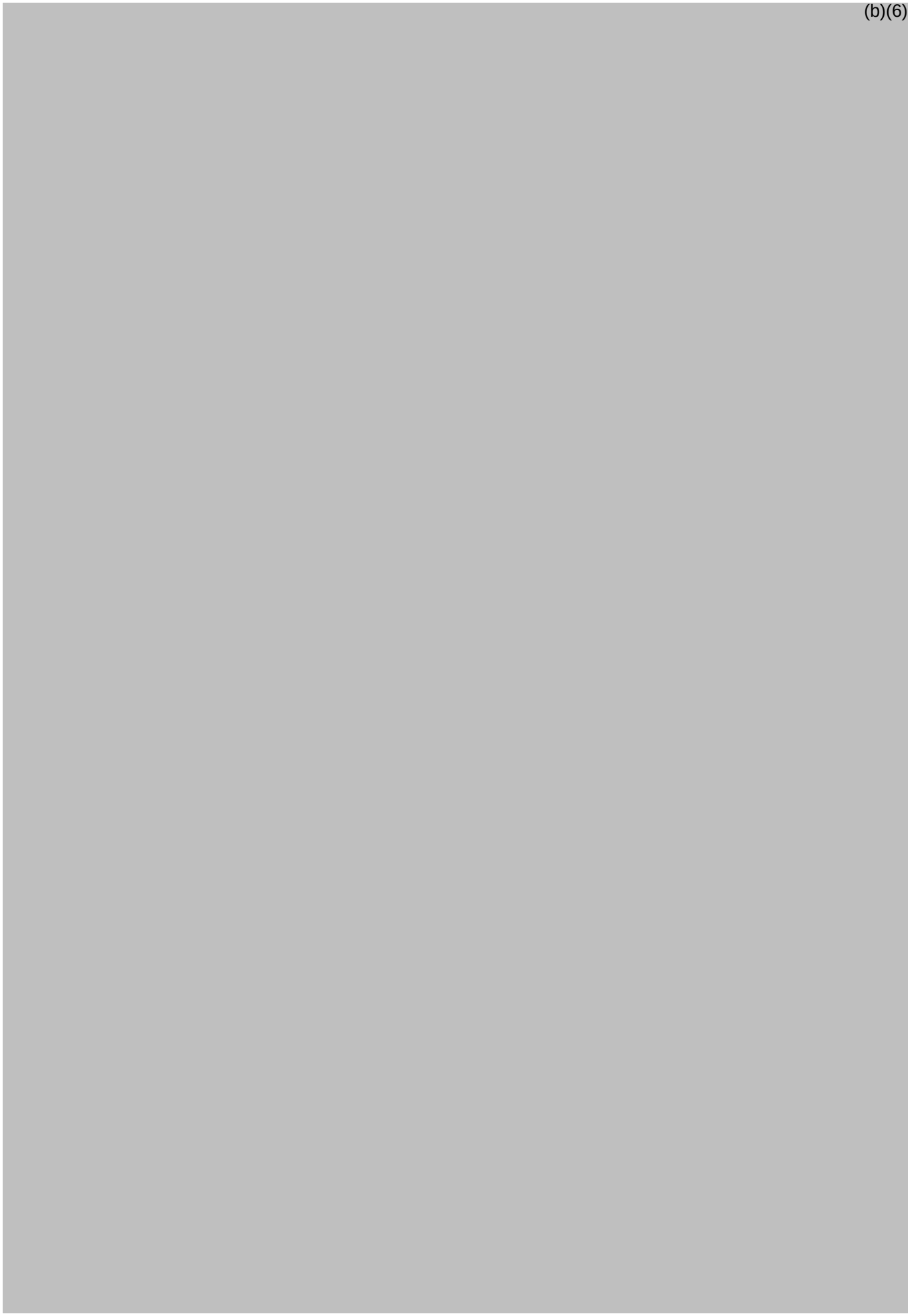
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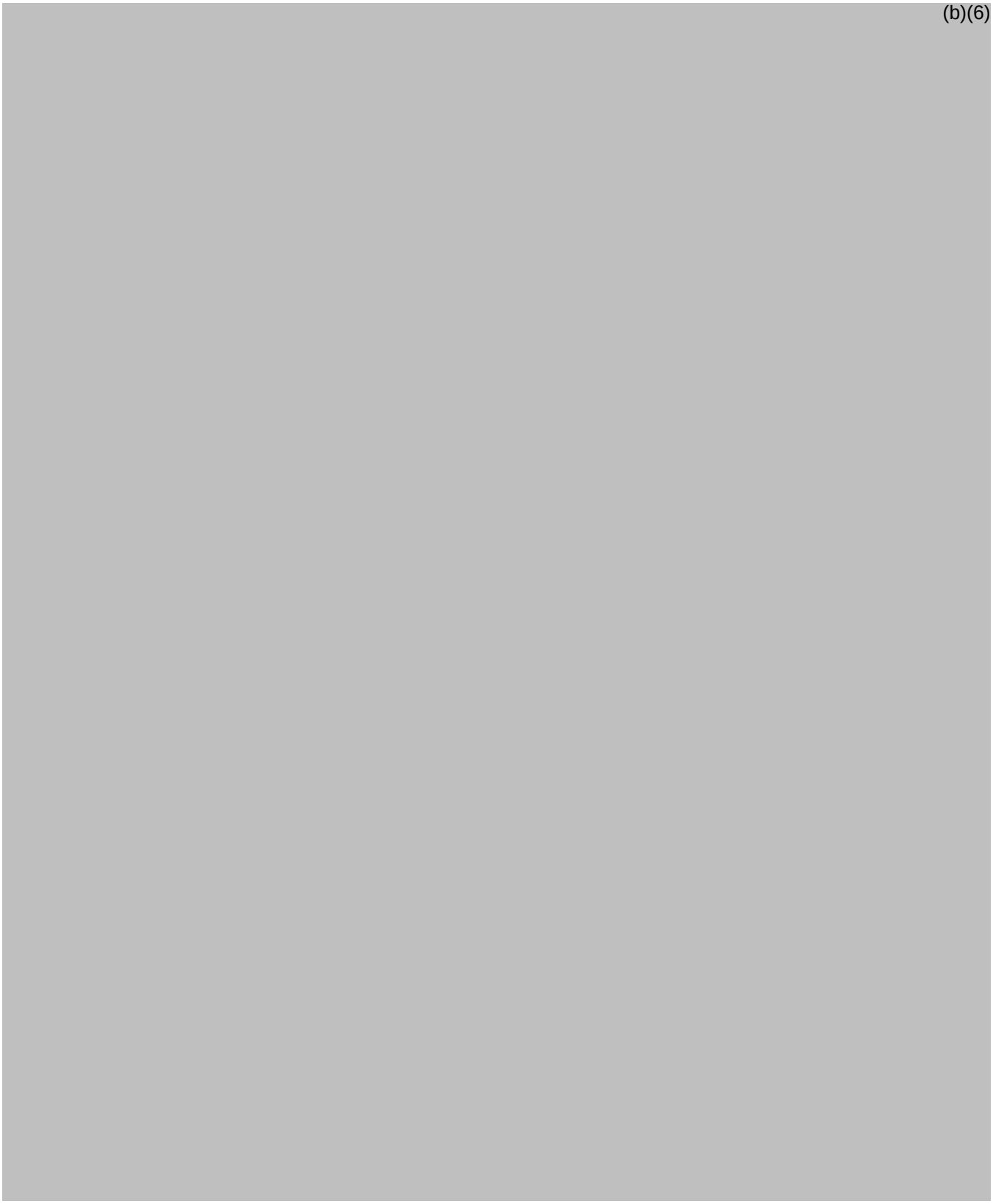

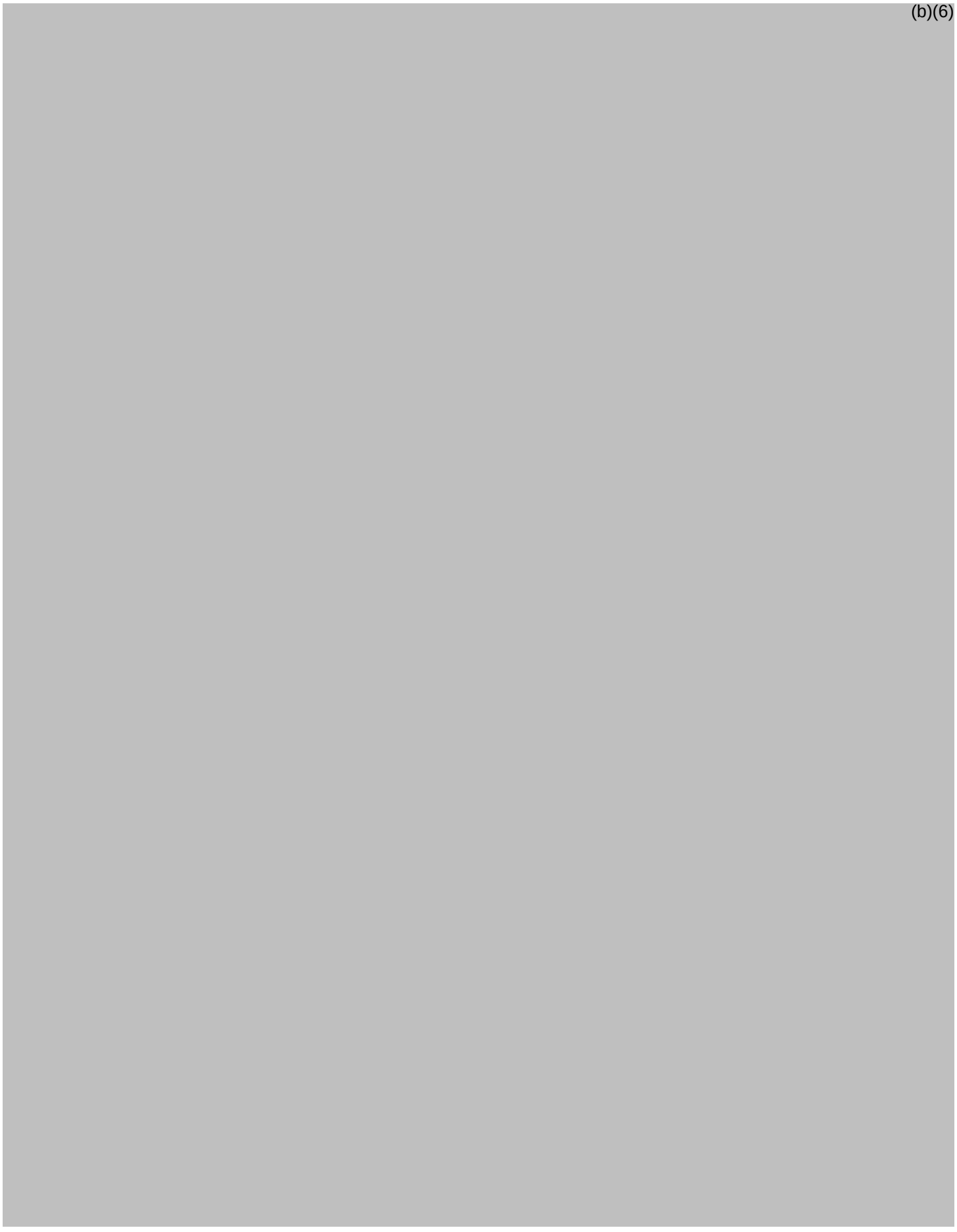
(b)(6)

From: Mahoney, Jill M.
To: Animalfood-premarket
Cc: Drozen, Melvin S.; Tomas Belloso; Conway, Charlotte
Subject: RE: [EXTERNAL] New GRN for "FeedKind"/Dried Methylococcus capsulatus
Date: Wednesday, May 25, 2022 12:11:12 PM

Hi Chelsea,

Calysta would prefer to receive communications regarding this submission via email.

Thank you,

Jill

From: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Sent: Wednesday, May 25, 2022 11:51 AM
To: Mahoney, Jill M. <mahoneyj@khlaw.com>
Cc: Drozen, Melvin S. <Drozen@khlaw.com>; Tomas Belloso <tbelloso@calysta.com>; Conway, Charlotte <Charlotte.Conway@fda.hhs.gov>; Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Subject: RE: [EXTERNAL] New GRN for "FeedKind"/Dried Methylococcus capsulatus

Hi Jill,

Thank you for the revised part 7.

How would Calysta prefer to receive our letters (email, paper, or both)? For previous submissions, we've sent them by email, but want to confirm Calysta's preference for this submission. We note the potential security risk with email and the letters containing confidential business information.

Thank you,

Chelsea

From: Mahoney, Jill M. <mahoneyj@khlaw.com>
Sent: Wednesday, May 25, 2022 11:16 AM
To: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Cc: Drozen, Melvin S. <Drozen@khlaw.com>; Tomas Belloso <tbelloso@calysta.com>; Conway, Charlotte <Charlotte.Conway@fda.hhs.gov>

Subject: RE: [EXTERNAL] New GRN for "FeedKind"/Dried Methylococcus capsulatus

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Hi Chelsea,

Thank you for your confirmation. Attached please find the revised Part 7 for Calysta's GRAS Notice for "FeedKind"/Dried Methylococcus capsulatus.

Please let us know if you have any questions.

Best,

Jill

From: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Sent: Wednesday, May 25, 2022 11:07 AM
To: Mahoney, Jill M. <mahoneyj@khlaw.com>
Cc: Drozen, Melvin S. <Drozen@khlaw.com>; Tomas Belloso <tbelloso@calysta.com>; Conway, Charlotte <Charlotte.Conway@fda.hhs.gov>; Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Subject: RE: [EXTERNAL] New GRN for "FeedKind"/Dried Methylococcus capsulatus

** EXTERNAL EMAIL **

Hi Jill,

Only Part 7 needs to be revised since, per your email, the references I noted yesterday are already cited in the appendices (as opposed to the other parts of the submission).

Thank you,

Chelsea

From: Mahoney, Jill M. <mahoneyj@khlaw.com>
Sent: Wednesday, May 25, 2022 10:44 AM
To: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Cc: Drozen, Melvin S. <Drozen@khlaw.com>; Tomas Belloso <tbelloso@calysta.com>; Conway, Charlotte <Charlotte.Conway@fda.hhs.gov>
Subject: RE: [EXTERNAL] New GRN for "FeedKind"/Dried Methylococcus capsulatus

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you

recognize the sender and know the content is safe.

Hi Chelsea,

We will send a revised Part 7 shortly. Can you please confirm that Part 7 is the only document that needs to be revised and submitted?

Thank you,

Jill

Jill M. Mahoney

Associate
KELLER AND HECKMAN LLP
tel: +1 202.434.4184| fax: +1 202.434.4646 | mahoney@khlaw.com
1001 G Street NW, Suite 500 West | Washington, DC 20001

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Keller and Heckman LLP's Food and Drug Practice is a Chambers USA recognized Band 1 firm.

From: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Sent: Wednesday, May 25, 2022 9:38 AM
To: Mahoney, Jill M. <mahoneyj@khlaw.com>
Cc: Drozen, Melvin S. <Drozen@khlaw.com>; Tomas Belloso <tbelloso@calysta.com>; Conway, Charlotte <Charlotte.Conway@fda.hhs.gov>; Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Subject: RE: [EXTERNAL] New GRN for "FeedKind"/Dried Methylococcus capsulatus

** EXTERNAL EMAIL **

Good morning Jill,

Thank you for the clarification regarding the citation of references. It would be helpful to have an updated Part 7 so that we have a complete list that matches the references included on the CD. With the updated Part 7, I will include your email below so its clear where citations of references

occur in the submission.

Kind regards,

Chelsea

From: Mahoney, Jill M. <mahoneyj@khlaw.com>
Sent: Wednesday, May 25, 2022 8:40 AM
To: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Cc: Drozen, Melvin S. <Drozen@khlaw.com>; Tomas Belloso <tbelloso@calysta.com>; Conway, Charlotte <Charlotte.Conway@fda.hhs.gov>; Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Subject: RE: [EXTERNAL] New GRN for "FeedKind"/Dried Methylococcus capsulatus

Hello Chelsea,

On behalf of Mel Drozen, please see our response to your May 24 email below.

We have reviewed the references listed in your email and can confirm that all of the references are included in the GRAS Notice for "FeedKind"/Dried Methylococcus capsulatus and should be considered as part of the evaluation of the submission. The following three references are already listed in Part 7 of the GRAS Notice:

- Abu El Ela (2008), is included on page 83 as "Aly MAEl E, Mahgoub IS, Nabawi M, Ahmed MAA (2008). Mercury monitoring and removal at gas-processing facilities: case study of Salam gas plant. SPE Proj. Facilit. Construct. 3(1): 1-9: https://www.researchgate.net/publication/250091182_Mercury_Monitoring_and_Removal_at_Gas-Processing_Facilities_Case_Study_of_Salam_Gas_Plant." The authors of the paper are listed as Mahmoud Abu El Ela Aly, Ismail Shaban Mahgoub, Mostafa Nabawi, Mohamed A. Aziem Ahmed.
- EPA 1995 4823-4495-6852 v.1, is included on page 88 as "US EPA (1995). Great Lakes water Quality Initiative technical Support Document for the Procedure to Determine Bioaccumulation Factors. Office of Water 4301. EPA-820-B95-005."
- USDA ARS User File (1994-1996 Intake Data), is included on page 88 as "Smiciklas-Wright H, Mitchell DC, Mickle SJ, Cook AJ, Goldman JD (2002). USDA 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII 1994-1996)."

We agree that the majority of the references are not included in Part 7, however, they are cited throughout and included in reference lists attached to Appendices 2 and 14:

- Referenced in Appendix 2 "n-Hexane, cyclohexane, benzene and toluene Safety Assessment" (see page 13 of appendix pdf):
    - Forlin et al (1984)
    - Kennish et al (1985)
    - Kennish et al (1988)
    - Luzanna (1998)
- Referenced Appendix 14 "Statement of Drs. Judith T. Zelikoff and Daniel Wierda" (see page 11 of appendix pdf):
    - Aschermann (2009) – Please note that the citation accidentally states 2010 instead of 2009
    - Barreto et al (2005)
    - Clausing and Bogh (2002)
    - Fei (2016)
    - Magadan (2015)
    - Martin (2017)
    - Molck et al (2002)
    - Nonaka (2000)
    - Norferm Sypplement to Dossier (2004)
    - Plomp (2017)
    - Rehberger (2017)

We would be happy to draft a revised Part 7 Reference List that includes all of the references cited and listed in Appendices 2 and 14. Please let us know if a revised Part 7 is necessary, or if the above identifying information is sufficient.

Additionally, we understand CVM is requesting a copy of Smiciklas-Wright (2002). As indicated above, Smiciklas-Wright (2002) was provided to CVM on the CD of references under the file name "USDA ARS User File (1994-1996 Intake Data)."

Please let us know if you have any questions.

Best,
Mel

From: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Sent: Tuesday, May 24, 2022 1:01 PM
To: Drozen, Melvin S. <Drozen@khlaw.com>
Cc: M. S. Tomas Belloso Ph. D. (tbelloso@calysta.com) <tbelloso@calysta.com>; Mahoney, Jill M. <mahoneyj@khlaw.com>; Conway, Charlotte <Charlotte.Conway@fda.hhs.gov>; Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Subject: RE: [EXTERNAL] New GRN for "FeedKind"/Dried Methylococcus capsulatus

** EXTERNAL EMAIL **

Dear Mr. Drozen,

Apologies for the delayed response as I was hoping to have more of an update to provide to you. We're nearing the completing of our initial, cursory pre-filing evaluation period, and are requesting clarification and more information regarding part 7 of the GRAS submission.

There are references included on the CD that are neither listed in part 7 nor referenced in the other parts of the submission. We're requesting clarification on whether or not these references should be included and considered as part of the evaluation of this submission? If so, it's requested they be added to part 7 as well as referenced in the other parts of the submission, where applicable. It may be easier to provide an update to all seven parts to capture these references. On the other hand, if these references are not part of this submission, please let us know.

The following are the references provided on CD, but not listed in part 7:

- Abu El Ela (2008)
- Aschermann (2009)
- Barreto et al (2005)
- Clausing and Bogh (2002)
- EPA 1995 4823-4495-6852 v.1
- Fei (2016)
- Forlin et al (1984)
- Kennish et al (1985)
- Kennish et al (1988)
- Luzanna (1998)
- Magadan (2015)
- Martin (2017)
- Molck et al (2002)
- Nonaka (2000)
- Norferm Sypplement to Dossier (2004) (note: filename is misspelled)
- Plomp (2017)
- Rehberger (2017)
- USDA ARS User File (1994-1996 Intake Data) – this reference is in a footnote on page 32, but not listed in part 7

Also, there is one reference [Smiciklas-Wright (2002)] that's listed in part 7 and references in the submission, but missing from the CD. Please provide a copy of this reference.

These items can be emailed to the animalfood-premarket@fda.hhs.gov mailbox if the Calysta is fine with items being sent by email. Also, if Calysta has a preference (email, paper, or both) for receiving our letters for this GRAS submission, we can issue them per their preference. We note the potential security risk with email and the letters containing confidential business information.

Kind regards,
Chelsea

From: Drozen, Melvin S. <Drozen@khlaw.com>
Sent: Wednesday, May 11, 2022 10:38 AM
To: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>; Cerrito, Chelsea <Chelsea.Cerrito@fda.hhs.gov>
Cc: M. S. Tomas Belloso Ph. D. (tbelloso@calysta.com) <tbelloso@calysta.com>; Mahoney, Jill M. <mahoneyj@khlaw.com>; Conway, Charlotte <Charlotte.Conway@fda.hhs.gov>
Subject: RE: [EXTERNAL] New GRN for "FeedKind"/Dried Methylococcus capsulatus

Hello Chelsea,

Can you please let us know where the filing review stands? Thanks very much. Regards. Mel Drozen.

REVISED Part 7 – List of supporting data and information

Calysta has disclosed all safety data of which it is aware and have found none that is inconsistent with the GRAS determination.

7.1 REVISED References

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(b) (4)

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Cerrito, Chelsea

From: Drozen, Melvin S. <Drozen@khlaw.com>
Sent: Tuesday, December 6, 2022 11:42 AM
To: Animalfood-premarket
Cc: Mahoney, Jill M.; Tomas M. Belloso M. S. Ph. D. (tbelloso@calysta.com); Pelonis, Evangelia C.
Subject: RE: [EXTERNAL] Status Update for GRAS Notice No. AGRN 60 - Dried Methylococcus capsulatus product

Hi Chelsea,

Sorry to not get back to you sooner, but I was out of town. We have confirmed that no data was intended to be included in the tab "Information for GRAS approval" in "Amendment Appendix 4. M capsulatus Heat Kill data." The tab was intended to be left blank.

Please let us know if you have any additional questions.

Best,
Mel.

From: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Sent: Tuesday, December 6, 2022 11:36 AM
To: Drozen, Melvin S. <Drozen@khlaw.com>
Cc: Mahoney, Jill M. <mahoneyj@khlaw.com>; Tomas M. Belloso M. S. Ph. D. (tbelloso@calysta.com) <tbelloso@calysta.com>; Pelonis, Evangelia C. <pelonis@khlaw.com>; Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Subject: RE: [EXTERNAL] Status Update for GRAS Notice No. AGRN 60 - Dried Methylococcus capsulatus product

Mr. Drozen,

Checking if Calysta has been able to confirm if the sheet noted in my email below in Amendment Appendix 4 is suppose to be blank or not?

Regards,
Chelsea

From: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Sent: Wednesday, November 30, 2022 11:14 AM
To: Drozen, Melvin S. <Drozen@khlaw.com>
Cc: Mahoney, Jill M. <mahoneyj@khlaw.com>; Tomas M. Belloso M. S. Ph. D. (tbelloso@calysta.com) <tbelloso@calysta.com>; Pelonis, Evangelia C. <pelonis@khlaw.com>; Animalfood-premarket <Animalfood-

premarket@fda.hhs.gov>
Subject: RE: [EXTERNAL] Status Update for GRAS Notice No. AGRN 60 - Dried Methylococcus capsulatus product

Dear Mr. Drozen,

A quick question regarding one of the appendices you provided as part of the amendment. In "Amendment Appendix 4. M capsulatus Heat Kill data", the sheet labeled "Information for GRAS approval" in the Excel spreadsheet is blank. I want to confirm that is the case or if there should be data captured there (we will need a new copy of the appendix, if so)?

Thank you,
Chelsea

From: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Sent: Tuesday, November 29, 2022 10:22 AM
To: Drozen, Melvin S. <Drozen@khlaw.com>
Cc: Mahoney, Jill M. <mahoneyj@khlaw.com>; Tomas M. Belloso M. S. Ph. D. (tbelloso@calysta.com) <tbelloso@calysta.com>; Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Subject: RE: [EXTERNAL] Status Update for GRAS Notice No. AGRN 60 - Dried Methylococcus capsulatus product

Dear Mr. Drozen,

Apologies for the delay in responding to your email. I'm acknowledging receipt of the amendment for GRAS Notice No. AGRN 60. If we have any questions, I will let you know.

Kind regards,
Chelsea

From: Drozen, Melvin S. <Drozen@khlaw.com>
Sent: Wednesday, November 23, 2022 2:58 PM
To: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Cc: Mahoney, Jill M. <mahoneyj@khlaw.com>; Tomas M. Belloso M. S. Ph. D. (tbelloso@calysta.com) <tbelloso@calysta.com>
Subject: [EXTERNAL] Status Update for GRAS Notice No. AGRN 60 - Dried Methylococcus capsulatus product

Dear Chelsea,

On behalf of Calysta, Inc., attached please find the Amendment to Animal GRAS Notice (AGRN) 60 for Dried *Methylococcus Capsulatus* product (hereinafter "FeedKind®"), which was submitted to CVM on March 22, 2022 and filed on June 2, 2022. The Amendment addresses the minor, clarifying questions raised by CVM in a November 9, 2022 email regarding (1) the manufacturing methods, (2) specifications, (3) analytical methods, (4) target animal safety, and (5) common or usual name.

The attached zip file contains (1) the Amendment to the GRASN, including a revised Part 7 reference list with five additional references highlighted in yellow, (2) appendices to the Amendment, and (3) copies of four of the new references – the remaining reference is available via hyperlink provided in the document.

Please let us know if you have any questions or if you have any difficulty accessing the materials. We look forward to receiving CVM's "no further questions" letter in the foreseeable future.

We hope you have a nice Thanksgiving holiday.

Best,
Mel

From: Drozen, Melvin S. <Drozen@khlaw.com>
Sent: Thursday, November 10, 2022 1:59 PM
To: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Cc: Mahoney, Jill M. <mahoneyj@khlaw.com>; Tomas Belloso <tbelloso@calysta.com>
Subject: RE: Status Update for GRAS Notice No. AGRN 60 - Dried Methylococcus capsulatus product

Dear Chelsea,

We have received your November 9 email below and CVM's questions. It is Calysta's intent to provide a response by the date requested, i.e., November 23. Best regards. Mel.

From: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Sent: Wednesday, November 9, 2022 2:48 PM
To: Drozen, Melvin S. <Drozen@khlaw.com>
Cc: Mahoney, Jill M. <mahoneyj@khlaw.com>; Tomas Belloso <tbelloso@calysta.com>; Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Subject: Status Update for GRAS Notice No. AGRN 60 - Dried Methylococcus capsulatus product

** EXTERNAL EMAIL **

Dear Mr. Drozen,

In regards to Calysta, Inc.'s GRAS notice for Dried *Methylococcus capsulatus* (*M. capsulatus*) product to be used as a source of protein in the diets of salmonid species, designated as GRAS Notice No. AGRN 60 that was filed on June 2, 2022. At this point in our evaluation of the notice, CVM has questions on the following sections that could potentially be addressed in a minor, clarifying amendment:

Manufacturing methods

- The GRAS notice uses the terms lots and batches. However, the notice does not contain definitions for these in terms of frequency (batch per time period). Because the manufacturing process is a (b) (4) (b) (4), the notifier should clarify how a lot or a batch is defined, for example, whether a "batch" or "lot" is the product harvested each day within a (b) (4) or from a different fermentation cycle.
- It is not clear from the information contained in the notice, the scale of the fermentation used to produce various data summarized in the notice (i.e. data on process control validation, batch analysis, and stability). For example, the notice does not describe whether a laboratory scale fermentation, pilot scale or production scale fermentation is used to validate the heat kill step for the viability of the production organism *M. capsulatus*. The notifier should clarify if the data on process control validation, batch analysis, and stability are generated by a pilot or lab-scale fermentation.

Specifications

The specification limits for aerobic plate count, yeast, mold, and nickel significantly deviate from the results of batch analysis summarized in Tables 4 and 9 in the notice. These specifications should be adjusted lower based on the provided batch analysis results to accurately reflect the expected levels.

Analytical methods

The notifier should provide a complete description of the analysis method and samples used to generate the internal data (Figure 3: *M. capsulatus* Heat Kill Curve) to demonstrate that *M. capsulatus* is entirely inactivated by the heat treatment process, and explain in more detail why the analysis method would not easily permit enumeration on a per lot basis. The method description should include the description of the culture method, method performance parameters (limit of detection, and statistical analysis), data and analysis, pictures of plates and representative calculations. The notifier should also provide a detailed description of the samples tested in this study. If the samples were not collected from a fermentation process before the heat kill step to produce the notified *M. capsulatus* product, the notifier should justify how the sample conditions are comparable to the real production conditions, such as cell density, so that the data presented in Figure 3 can be used to support the conclusion that *M. capsulatus* is entirely inactivated by the heat treatment process.

Target Animal Safety

In Appendix 10, Shelf Life Testing of FeedKind, we noticed the amount of spermidine in the samples to be high, ranging from 3000 ppm to 5000 ppm. Thus, if the substance is used at 18% in the diet of salmonid fish, the amount of spermidine in the diet could be up to 900 mg of sperimidine per kilogram of feed (considering a content of 5000 ppm of spermidine in the substance). Please explain how you have substantiated that this amount of spermidine will not pose a target animal safety concern for salmonid fish.

Common or Usual Name

The notified substance is identified as "dried *Methylococcus capsulatus* product" and described as a culture of methanotrophic and heterotrophic microbial consortia that is 90% *M. capsulatus*. Because the notified substance is not entirely a biomass of *M. capsulatus*, the notifier should propose a different name to describe the notified substance.

The notifier may provide an amendment to address the questions and comments in this email. The notifier should send this amendment to animalfood-premarket@fda.hhs.gov within the next two weeks, which is no later than Wednesday, November 23, 2022. Alternatively, the notifier may send a letter asking CVM to cease to evaluate the GRAS Notice. If no response is received, CVM will proceed with evaluation of the notice.

Thank you,
Chelsea

Chelsea Cerrito, MAS
Animal Scientist, Division of Animal Food Ingredients (DAFI)

Center for Veterinary Medicine
Office of Surveillance and Compliance
U.S. Food and Drug Administration
Tel: 240-402-6729
Personal e-mail address: Chelsea.Cerrito@fda.hhs.gov
To schedule a meeting with DAFI, please e-mail: animalfood-premarket@fda.hhs.gov

AMENDMENT TO ANIMAL GRAS NOTICE 60 FOR DRIED *METHYLOCOCCUS CAPSULATUS* PRODUCT

Submitted by: Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington, DC 20001

On behalf of our client:

Calysta, Inc.
1900 Alameda de las Pulgas
Suite 200
San Mateo, CA 94403

November 23, 2022

I. INTRODUCTION

The purpose of this amendment is to address the minor, clarifying questions raised by the U.S. Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) in a November 9, 2022 email regarding the March 22, 2022 submission of the Generally Recognized As Safe (GRAS) Notice for Calysta, Inc.'s Dried *Methylococcus capsulatus* Product (hereinafter "FeedKind®"), which was filed on June 2, 2022. For clarity and convenience, we have repeated CVM's questions in **bold** below, followed by our responses.

II. MANUFACTURING METHODS

1. **The GRAS notice uses the terms lots and batches. However, the notice does not contain definitions for these in terms of frequency (batch per time period). Because the manufacturing process is a** (b) (4) **cycle, the notifier should clarify how a lot or a batch is defined, for example, whether a "batch" or "lot" is the product harvested each day within a** (b) (4) **cycle or from a different fermentation cycle.**

In Animal GRAS Notice (AGRN) 60, "batch" and "lot" are used interchangeably. A "batch" / (b) (4) run typically lasts (b) (4) but may be terminated early depending on operational requirements. Calysta is currently planning TPP20 and will continue to add runs as needed and label them as such.

2. **It is not clear from the information contained in the notice, the scale of the fermentation used to produce various data summarized in the notice (i.e. data on process control validation, batch analysis, and stability). For example, the notice does not describe whether a laboratory scale fermentation, pilot scale or production scale fermentation is used to validate the heat kill step for the viability of the production organism *M. capsulatus*. The notifier should clarify if the data on process control validation, batch analysis, and stability are generated by a pilot or lab-scale fermentation.**

The majority of data contained in AGRN 60 are from pilot scale fermentation, including the process control validation (Table 4), batch analysis data (Table 9), and stability data (Tables 12-14). Data from a laboratory scale fermentation are presented in Table 1, in the columns labeled "Data Reviewed in Original EFSA Approval" (all lab scale) and "All Batches" (combination of lab and pilot scale). None of the data submitted comes from production scale fermentation. For Calysta's purposes, (b) (4) (b) (4)

III. SPECIFICATIONS

1. **The specification limits for aerobic plate count, yeast, mold, and nickel significantly deviate from the results of batch analysis summarized in Tables 4 and 9 in the notice. These specifications should be adjusted lower based on the provided batch analysis results to accurately reflect the expected levels.**

Calysta agrees to reduce the specifications for both yeast and mold from (b) (4) cfu/g. Calysta also agrees to lower the nickel specification from (b) (4) Calysta believes these limits are in line with the previously submitted data and provide sufficient safety margins for the listed analytes.

However, Calysta believes that the aerobic plate count specification needs to remain at (b) (4) cfu/g. The data in Table 4 were obtained immediately after the heat kill step and generated and submitted as validation of the heat treatment step as a kill step for the production organisms. Following the heat treatment process, the product is stored, concentrated and dried using (b) (4) The overall aerobic plate count defined in Calysta's specification is a reflection of the capability of these (b) (4) inevitably producing some residual microbiological levels even with sanitary design, validated clean-in-place and robust prerequisite programs. These levels are well understood and, therefore, Calysta has set the specification in line with the specification set by the USDA for Dry Whole Milk and Instant Nonfat Dry Milk - U.S Extra Grade of NMT (b) (4) aerobic plate count. Attached as "Amendment Appendix 1. United States Standards for Grades of Dry Whole Milk" and "Amendment Appendix 2. United States Standards for Instant Nonfat Dry Milk."

Table 9 is a reflection of what the Calysta production and sanitation teams have been able to achieve in the cleaning and management of the system. As Calysta gathers more experience over the years, the plan is to continue to review and analyze data and set internal microbiological specifications based on the system capability. However, Calysta believes that the current specification of (b) (4) is a realistic specification, and would not suggest or indicate the product is adulterated or unsafe. This is supported by the fact that Table 4 clearly indicates that the production organism is completely inactivated by the heat treatment step, and, that Calysta has set zero as pathogenic bacteria specifications.

For convenience, a revised specification table is provided below.

REVISED Table 8. FeedKind® Specifications

Specifications				
Chemical Composition	**Minimum**	**Maximum**	**Units**	**Test Method**
Crude Protein	(b) (4)		% dry weight	DUMAS method

Crude Fat	(b) (4)	% dry weight	modified Weibull Acid Hydrolysis Method
Crude Fiber		% dry weight	AOCS Ba 6a-05, Ba 6-84 AOAC 962.09, S 1022 using Gravimetry
Ash[1]		% dry weight	AOAC 942.05, S 1024 using Gravimetry
Moisture		w/w	AOAC 934.01, 930.15, S 1024 using Gravimetry
Nickel		*mg/kg*	ICP-MS Internal Method[2]
Mercury		*mg/kg*	ICP-MS Internal Method[3]
Microbiological Limits	**Limits**		**Test Method**
Mesophilic Aerobic Plate Count[4]	(b) (4)		EN ISO 4833:2013
Mold			AOAC 997.02; FDA/BAM Chapter 18
Yeast			AOAC 997.02; FDA/BAM Chapter 18
Salmonella			EN ISO 6579-2:2017

[1] Appendix 5 contains analyses of 289 lots. These data show that a true average value for ash is (b) (4) with a standard deviation (b) (4) The average plus (b) (4) Ash fluctuates predictably due to fermentation stage and productivity. Startup and low productivity levels in the fermenter deliver higher ash level while high productivity or steady state operations have lower ash levels. The representative samples had low ash because they happened to be taken during periods of high productivity. Given that ash is primarily salts and minerals present in the media, and higher ash is not seen as a health risk because Calysta concurrently monitors for heavy metals and contaminants directly, we believe that leaving the ash specification at (b) (4) is appropriate. Ash is not used as a proxy for any other measurements.

[2] Sciantec has developed an in-house validation method for the detection measurement of nickel with an LOD of 0.1mg/kg in animal feed. The method and validation summary are included in Appendices 6 and 7. Appendix 8 details Sciantec's validation of various analytical methods.

[3] The method used to determine the mercury content is validated and accredited by (b) (4) The method summary is included in Appendix 8 for detection of mercury at an LOD of 0.01mg/kg.

[4] Calysta has previously tested 276 separate lots of FeedKind® produced during research and development phases to optimize the production process, the results of which were previously provided to FDA. Of these lots, 8 results were extremely high (> 500,000 cfu/g). Reference to these production lots have been removed as they were produced with the old production process prior to introduction of the new direct steam treatment and as such are not relevant to this notice.

Listeria	(b) (4)	EN ISO 11290-1:2017

IV. ANALYTICAL METHODS

1. **The notifier should provide a complete description of the analysis method and samples used to generate the internal data (Figure 3: M. capsulatus Heat Kill Curve) to demonstrate that *M. capsulatus* is entirely inactivated by the heat treatment process, and explain in more detail why the analysis method would not easily permit enumeration on a per lot basis. The method description should include the description of the culture method, method performance parameters (limit of detection, and statistical analysis), data and analysis, pictures of plates and representative calculations. The notifier should also provide a detailed description of the samples tested in this study. If the samples were not collected from a fermentation process before the heat kill step to produce the notified *M. capsulatus* product, the notifier should justify how the sample conditions are comparable to the real production conditions, such as cell density, so that the data presented in Figure 3 can be used to support the conclusion that M. capsulatus is entirely inactivated by the heat treatment process.**

Attached as "Amendment Appendix 3. SOP-M-14 Enumeration of Methylococcus capsulatus," please find the full culture method employed by Calysta to culture *M. capsulatus* and generate the heat kill data. As is detailed in the method, it is impractical to conduct enumeration on a per batch basis due to the growth condition requirements including:

(b) (4)

For the heat kill curve data (Figure 3), cultures of *M. capsulatus* were grown at (b) (4)

(b) (4)

(b) (4)

(b) (4)

The average and standard deviation of the results (CFU/ml and CFU/ml/OD) for each temperature were determined.

Attached as "Amendment Appendix 4. M capsulatus Heat Kill data," please find an excel file which includes details and raw data from the *M. capsulatus* heat kill experiment reported in Figure 3 of AGRN 60. While this analysis was conducted using laboratory cultures rather than production fermentation lots, Calysta attempted to recreate the conditions of production fermentation (time, temperature, cell density, media makeup, etc.) as closely as possible. The data presented in Figure 3 (heat kill curve) as well as in Table 4 (heat kill step validation) show that the production organisms are completely inactivated by the production conditions.

V. TARGET ANIMAL SAFETY

1. **In Appendix 10, Shelf Life Testing of FeedKind, we noticed the amount of spermidine in the samples to be high, ranging from 3000 ppm to 5000 ppm. Thus, if the substance is used at 18% in the diet of salmonid fish, the amount of spermidine in the diet could be up to 900 mg of sperimidine per kilogram of feed (considering a content of 5000 ppm of spermidine in the substance). Please explain how you have substantiated that this amount of spermidine will not pose a target animal safety concern for salmonid fish.**

Biogenic amines are organic, basic, nitrogenous compounds of low molecular weight, mainly formed by decarboxylation of amino acids (see Figure 1[6] below).

[6] (b) (4)

(b) (4)

The 2-D chemical structures of the biogenic amines commonly reported in the pertinent scientific literature are depicted in Figure 2 below.

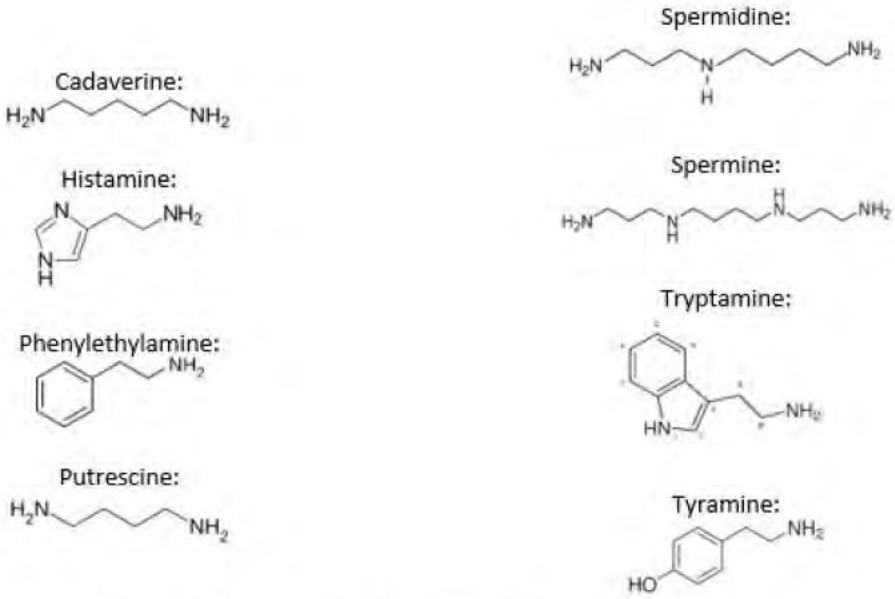

Figure 2. Molecular Structures of Biogenic Amines

Based on chemical structures, these substances can be classified as aliphatic (i.e., cadaverine, putrescine, spermidine, spermine), heterocyclic (e.g., histamine, tryptamine) or aromatic (e.g., phenylethylamine, tyramine). Based on the number of amine groups, these substances can be classified as monoamines (e.g., tyramine), diamines (e.g., cadaverine, histamine, putrescine) or polyamines (e.g., spermidine and spermine). The polyamines synthesized by mammals include the triamine spermidine, the tetramine spermine, and their precursor putrescine, which are synthesized through the anabolic decarboxylation of ornithine and methionine.[7] These native polyamines, sometimes referred to in the literature as the "mammalian polyamines," play vital roles in the growth and development of all mammalian species.

Generally, the polyamines are essential for cell proliferation. The biosynthesis of polyamines is stimulated by regenerative and growth-promoting hormones and, thus, the levels of polyamines are higher in rapidly growing tissues.

Biogenic amines have important biological functions in animals and, thus, there is value to having appropriate levels of these compounds in feed.

On the other hand, spoilage of foods and feeds is associated with the high levels of biogenic amines that are produced by several species of spoilage microorganisms. The richest sources of biogenic amines in the diet include byproducts that have undergone some degree of spoilage, including meat and bone meals, blood meal, feather meal, poultry byproduct meal and fish meal.[8] Fish meal is by far the predominating source of protein in fish foods used in the aquaculture of salmonids and other food-producing fish. Stale fish meal used for such purposes has been reported to be associated with reduced growth and development of fish and shellfish, which are, thus, associated with the high content of biogenic amines in stale fish meals. These observations have been studied and reported in several published scientific research articles. The research is relevant because FeedKind® is intended to be used as a replacement for fish meal in salmonid foods. The relevant studies demonstrate that biogenic amines in fish meal at levels as high as or greater than those measured in FeedKind® may serve as an indicator of the freshness of fish meal used to manufacture fish foods but adverse health effects in salmonids or other aquatic species ingesting diets containing stale fish meal are not attributable to the toxicity of the biogenic amines in these diets. Thus, there is no reasonable expectation of harm to salmonids from exposure to spermidine and other biogenic amines in diets containing up to 18% FeedKind®. The key studies are summarized below.

(b) (4)

[7] (b) (4)

[8] (b) (4)

(b) (4) al.[9] Each diet contained 61.31% fish meal. Each biogenic amine was added to the diet containing the fresh fish meal to approximate the corresponding levels in the diet containing the stale fish meal. The highest total measured concentrations of biogenic amines were 4577 mg/kg in the diet containing fresh fish meal to which cadaverine, histamine, putrescine and tyramine were added and 5592 mg/kg in the diet containing stale fish meal. See Amendment Table 1.

Amendment Table 1. Biogenic Amines in Diets Tested by (b) (4) *et al.* (2000)

Diet #	Fish Meal Source	Polyamine(s) Added	Measured Dietary Biogenic Amine Concentrations (mg/kg)				
			Cadaverine (C)	Histamine (H)	Putrescine (P)	Tyramine (T)	**Total**
1	Fresh	None	104	48	67	44	**263**
2	Stale	None	2156	1742	828	866	**5592**
3	Fresh	CHPT*	1649	1584	624	720	**4577**
4	Fresh	CPT	1724	61	627	703	**3115**
5	Fresh	HPT	137	1688	727	749	**3301**
6	Fresh	CH	1424	1423	76	62	**2985**

*C = cadaverine; H = histamine; P = putrescine; T = tyramine

The salmon fed the diet containing the stale fish meal exhibited statistically-significantly reduced food consumption (represented by offered feed), body weight, growth and specific growth rate (SGR) and elevated feed conversion ratio (FCR) compared with the salmon fed the diet containing the fresh fish meal. The feed intake and growth of the animals were severely affected by the inclusion of the stale fish meal in the diet, without affecting the FCR, compared with those fed the diet containing fresh fish meal. The growth rate of salmon fed diet containing stale fish meal was less than 50% of the growth rate of salmon fed the diet containing fresh fish meal.

In contrast, there were no statistically-significant differences observed in any of these parameters among the salmon fed diets containing fresh fish meal plus biogenic amines and the diet containing only fresh fish meal. On the contrary, the addition of biogenic amines appeared to improve feed consumption and FCR of the fish fed the fresh fish meal (although the differences were not statistically significant).

Gross and histopathological examinations revealed no statistically significant effect on the incidences of lesions in the pancreas, muscle or kidneys of the animals fed Diet 2 (stale fish meal) or Diet 3 (fresh fish meal plus all 4 biogenic amines added), compared with those fed Diet 1 (fresh fish meal). However, the salmon fed Diet 2 exhibited statistically significantly elevated incidences of lesions of the liver and intestines and in the severity grade of liver lesions.

[9] (b) (4)

The authors noted that the reduced production performance of the fish fed the diet containing stale herring meal, including effects on growth and FCR, is attributable to compounds formed during the storage, handling and/or processing of stale fish, which may reduce the palatability and essential amino acid content of the diet. These factors probably play a relatively minor role. On the other hand, some of the compounds in stale fish meal may cause pathological changes in vital organs, which result in reduced growth. The adverse effects in fish fed stale fish meal are attributable principally to the formation of such toxic compounds in the meal.

Overall, the results of this study demonstrated conclusively that, while the level of biogenic amines may serve as a quality criterion indicating the freshness of fish meals, the adverse effects of stale fish meal on the health of the animals is not attributable to the biogenic amines.

(b) (4)

The highest total measured concentrations of biogenic amines were 1581 mg/kg in the diet containing fresh fish meal to which cadaverine, histamine, putrescine and tyramine were added and 1985 mg/kg in the diet containing stale fish meal. See Amendment Table 2.

Amendment Table 2. Biogenic Amines in Diets Tested by (b) (4)

Diet #	Fish Meal Source	Polyamine(s) Added	Measured Dietary Biogenic Amine Concentrations (mg/kg)				
			Cadaverine (C)	Histamine (H)	Putrescine (P)	Tyramine (T)	**Total**
1	Fresh	None	17	11	14	6	**48**
2	Stale	None	608	653	426	298	**1985**
3	Fresh	CHPT*	538	632	331	80	**1581**
4	Fresh	CPT	0	561	323	241	**1125**
5	Fresh	HPT	549	69	446	261	**1325**
6	Fresh	CH	559	620	5	0	**1184**

The shrimp fed the diet containing stale fish meal exhibited statistically-significantly reduced survival, feed consumption, and final biomass compared with those fed the diet containing fresh fish meal. Shrimp fed the diet containing stale fish meal exhibited statistically-significantly

[10] (b) (4)

lower Individual weight and weight gain compared with shrimp fed Diet 6 (i.e., containing fresh fish meal plus added cadaverine and histamine), which had statistically-significant increases in feed consumption, wet weight, and final biomass, with no effect on FCR, compared with shrimp fed Diet 1 (i.e., containing fresh fish meal). None of the other diets (i.e., Diet 3, 4, and 5) produced discernible effects on any of these parameters.

There were no statistically-significant differences in polyamine tissue concentrations (hepatopancreas and remaining whole body) across the groups, although cadaverine concentrations appeared to be higher in shrimp fed diet containing stale fish meal and spermidine concentration tended to by higher in shrimp fed the diets supplemented with combinations of histamine, putrescine and tyramine (i.e., diet 3 and 5). The authors noted that spermidine is a metabolite of putrescine.

In addition, (b) (4) As well, the increase in the weight of shrimp fed Diet 6 is in line with studies in poultry reporting analogous effects when the feed was supplemented with up to but less than 2000 mg/kg spermidine, putrescine and histamine.

Overall, the study reported by (b) (4) clearly demonstrated that fish meal made from stale fish contains a factor or factors toxic to shrimp but that these factors are not biogenic amines, which had no adverse effects on the shrimp. These authors also noted that likely candidate causative factor(s) for the adverse effects of stale fish meal in the diets are endotoxic lipopolysaccharides (LPS) or other endotoxins of the gram-negative bacterial species responsible for producing biogenic amines from the amino acids.

In contrast to the spoilage microorganisms responsible for biosynthesizing biogenic amines, the microbial consortium used to produce FeedKind® and, thus, FeedKind® do not contain potentially harmful LPS or other endotoxins (see AGRN 60 Section 6.1.1. Safety of the microorganisms).

Furthermore, the biogenic amines measured in FeedKind® are not the result of spoilage. AGRN 60 Appendix 10 “Shelf Life Testing of FeedKind® Interim Report” Supplemental FeedKind® Shelf Life Report presents the levels of 6 biogenic amines in 8 randomly selected batches of FeedKind® that were stored under controlled conditions for 52 weeks. Samples of each batch were collected for analysis on weeks 0, 4, 8, 26, and 52 of storage and 6 of the 8 batches were sampled on week 39 as well.

The storage data presented in Appendix 10 indicate that the biogenic amines in FeedKind® did not increase during the 52-week storage period, contrary to the increase in the concentrations of these substances that would be expected from the activity of spoilage microorganisms over time. Thus, the presence of biogenic amines in FeedKind® is attributable to the biosynthesis of biogenic amines (especially spermidine) by the bacteria of the production consortium rather than to product spoilage over the storage period.

The average mean concentrations of total biogenic amines and spermidine across the storage period is 4003 mg/kg and 3839 mg/kg, respectively. The intended maximum FeedKind® inclusion rate in salmonid foods is 18%. Assuming, conservatively, that FeedKind® never contains more than 5000 mg/kg biogenic amines, including spermidine, the maximum biogenic amines concentrations in diets containing no more than 18% FeedKind would be 900 mg/kg. This value is more than 4-fold to 5-fold lower than the concentrations of total biogenic amines measured in Diets 3, 4, 5 and 6 (i.e., 2984 to 4577 mg/kg) tested in salmon by (b) (4). (2000) and is less than the total biogenic amines in Diets 3, 4, 5 and 6 (i.e., 1125 to 1581 mg/kg) tested in shrimp by (b) (4)

As noted above, the total concentrations of biogenic amines in Diets 3, 4, 5 and 6 tested in salmon and in shrimp at concentrations substantially greater than those measured in FeedKind® revealed no adverse effects in these studies. Thus, there is no reasonable expectation of harm to salmonids fed diets containing up to 18% FeedKind®. This conclusion is consistent with, and supported by, the results of the safety studies, which are summarized and evaluated in Section 6 (Narrative) of AGRN 60.

VI. COMMON OR USUAL NAME

1. **The notified substance is identified as "dried Methylococcus capsulatus product" and described as a culture of methanotrophic and heterotrophic microbial consortia that is 90% M. capsulatus. Because the notified substance is not entirely a biomass of M. capsulatus, the notifier should propose a different name to describe the notified substance.**

As CVM knows, the Federal Food, Drug, & Cosmetic Act (FD&C Act) requires that the name of a food ingredient not be false or misleading. Specifically, section 403(i) requires that finished foods and food ingredients be identified by their "common or usual name" on the product label. These requirements are codified in FDA's regulations at 21 CFR §§ 501.3 and 501.4 ("Identity labeling of animal food in packaged form" and "Animal Food; designation of ingredients"). 21 CFR 502.5(a) specifically provides that:

> The common or usual name of a food, which may be a coined term, shall accurately identify or describe, in as simple and direct terms as possible, the basic nature of the food or its characterizing properties or ingredients. The name shall be uniform among all identical or similar products and may not be confusingly similar to the name of any other food that is not reasonably encompassed within the same name. Each class or subclass of food shall be given its own common or usual name that states, in clear terms, what it is in a way that distinguishes it from different foods.

[11] For example, 2984 mg/kg ÷ 721 mg/kg = 4.14.

Accordingly, a common or usual name must be descriptive, uniform, and unique in as simple and direct terms as possible.

The name "Dried *Methylococcus capsulatus* Product" describes the basic nature of the ingredient, however, as CVM noted, while *M. capsulatus* constitutes the majority of the microbial consortia, the ingredient is not entirely a biomass of *M. capsulatus*. Therefore, Calysta proposes the name "Microbial protein from *Methylococcus*."

The proposed name clearly describes the ingredient, which contains (b) (4) protein, as a protein ingredient produced from microbial sources, and includes the identity of the dominant strain. Indeed, "Microbial protein from *Methylococcus*" appears to be in line with precedent set by CVM for shortened common or usual names for other notified substances. For instance, "*Euphausia superba* (krill) meal" is the notified substance for AGRN 30 and, as indicated in FDA's response letter, is recognized by the shortened common name "krill meal." Similarly, "Marine microalgae oil from *Schizochytrium sp*." is the notified substance for AGRN 36 and may be recognized by the shortened name "marine microalgae oil." Notably, neither example includes the genus or species of the substance in the common name, but Calysta's proposal provides even greater specificity by identifying the ingredient's dominant fermentation strain.

We believe that the proposed name "Microbial protein from *Methylococcus*" accurately describes the identity of the ingredient in as simple and direct terms as possible, in accordance with the FD&C Act and the FDA's implementing regulations.

* * *

We trust that the above information fully responds to CVM's questions, and look forward to receiving CVM's "no further questions" letter in the foreseeable future.

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Amendment Appendices

1. **Amendment Appendix 1. United States Standards for Grades of Dry Whole Milk**
2. **Amendment Appendix 2. United States Standards for Instant Nonfat Dry Milk**
3. **Amendment Appendix 3. SOP-M-14 Enumeration of Methylococcus capsulatus**
4. **Amendment Appendix 4. M capsulatus Heat Kill data**

United States
Department of
Agriculture

Agricultural
Marketing
Service

Dairy
Programs

United States Standards for Grades of Dry Whole Milk

Effective April 13, 2001

United States
Department of
Agriculture

Agricultural
Marketing
Service

Dairy
Programs

United States Standards for Instant Nonfat Dry Milk

Effective February 5, 2001
Reviewed June, 2013

United States Standards for Instant Nonfat Dry Milk [1]

Definitions

§ 58.2750 Instant nonfat dry milk.

(a) Instant nonfat dry milk is nonfat dry milk which has been produced in such a manner as to substantially improve its dispersing and reconstitution characteristics over that produced by the conventional processes. Instant nonfat dry milk covered by these standards shall not contain dry buttermilk, dry whey, or products other than nonfat dry milk, except that lactose may be added as a processing aid during instantizing. The instant nonfat dry milk shall not contain any added preservatives, neutralizing agents, or other chemicals. If lactose is used, the amount of lactose shall be the minimum required to produce the desired effect, but in no case shall the amount exceed 2.0 percent of the weight of the nonfat dry milk. If instant nonfat dry milk is fortified with vitamin A, and the product is reconstituted in accordance with the label directions, each quart of the reconstituted product shall contain 2000 International Units thereof. If instant nonfat dry milk is fortified with vitamin D, and the product is reconstituted in accordance with the label directions, each quart of the reconstituted product shall contain 400 International Units thereof.

(b) "Nonfat dry milk" is the product obtained by the removal of only water from pasteurized skim milk. It contains not more than 5 percent by weight of moisture and not more than 1 ½ percent by weight of milkfat and it conforms to the applicable provisions of 21 CFR part 131, "Milk and Cream" as issued by the Food and Drug Administration. Nonfat dry milk shall not contain nor be derived from dry buttermilk, dry whey, or products other than skim milk, and shall not contain any added preservative, neutralizing agent, or other chemical.

U.S. Grade

§ 58.2751 Nomenclature of the U.S. Grade

The nomenclature of the U.S. grade is U.S. Extra.

[1] Compliance with these standards does not excuse failure to comply with the provisions of the Federal Food, Drug and Cosmetic Act.

1. Purpose

This procedure provides detailed instructions for the enumeration of *Methylococcus capsulatus*.

2. Applicability

This procedure is applicable to all Calysta sites worldwide.

3. Scope

The enumeration of *Methylococcus capsulatus* is a quantitative assay which uses a combination of serial dilutions and spread plating techniques to establish viable bacterial cell counts.

4. Responsibilities

4.1 It is the responsibility of every individual to adhere to the procedure described in this document for the enumeration of *Methylococcus capsulatus*.

4.2 It is the responsibility of the individual to use proper safety guidelines when handling all chemicals.

4.3 It is the responsibility of all staff to be trained in, understand, and follow this procedure.

4.4 It is the responsibility of all Team Managers to ensure their teams are trained fully in the procedure and are following it.

5. Procedure

5.1 Equipment and Materials

(b) (4)

(b) (4)

(b) (4)

(b) (4)

6. References

Basic operation of the MSC Advantage Class II Biological Safety Cabinet Work Instruction

Revision Status

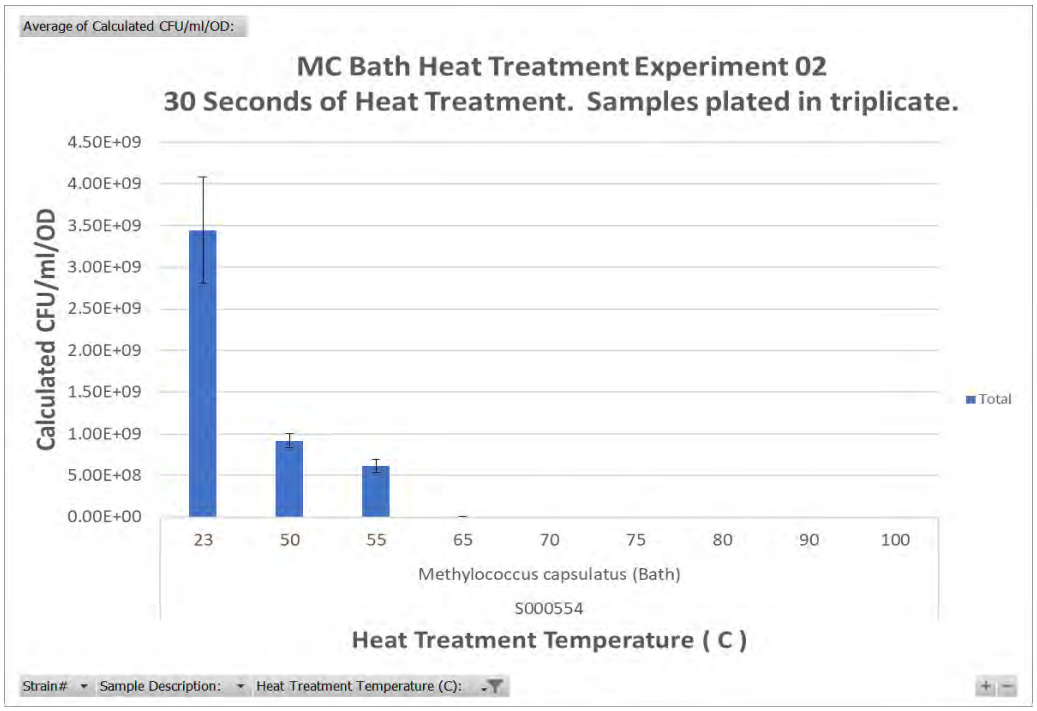
Revision	Date	Reason	Revised by	Approved by
1	13 Nov 22	New Document		(b)(6)

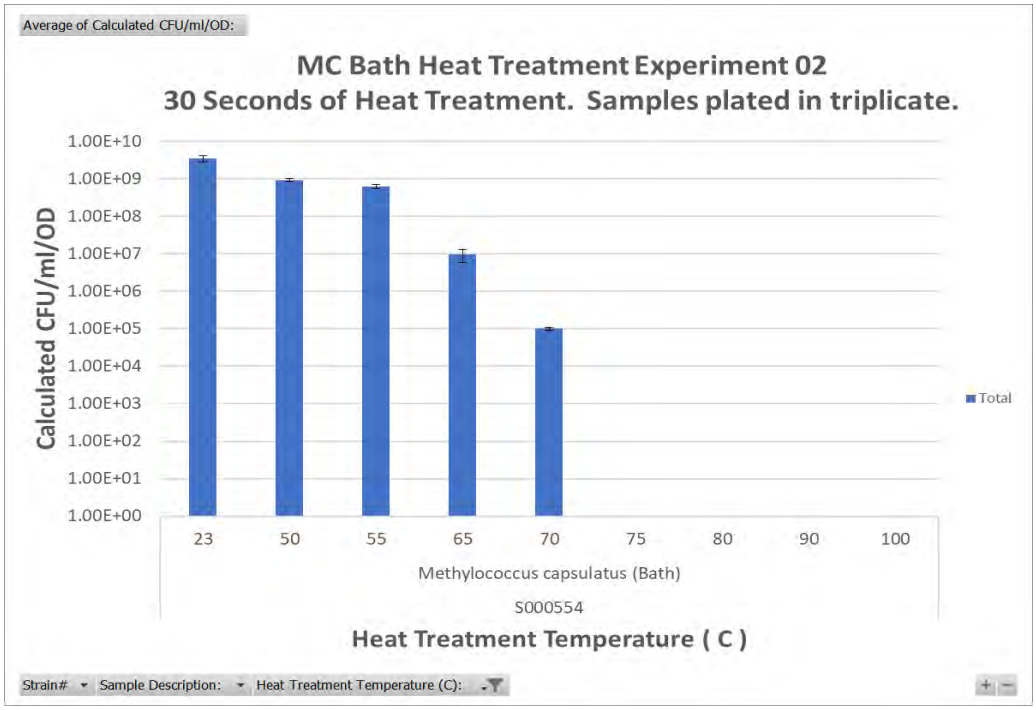
Amendment Appendix 4. M capsulatus Heat Kill data.xlsx - see M-000122-T-0003_sub_002

Note: Notifier has confirmed that the sheet labeled "Information for GRAS approval" in Excel sheet is blank

New References

1. (b) (4)
2.
3.
4.



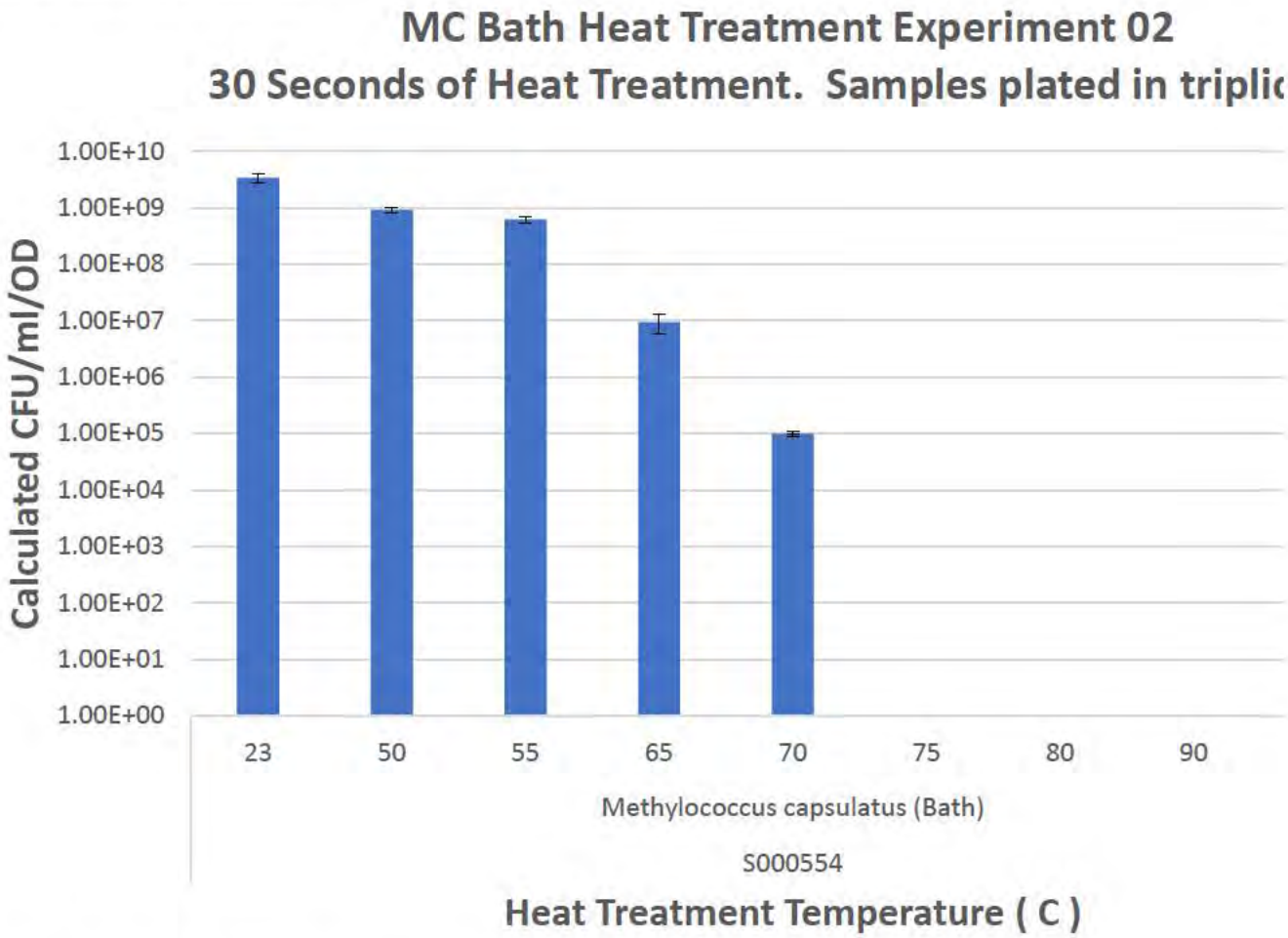


Row Labels	StdDev of Calculated CFU/ml/OD:
Methylococcus capsulatus (Bath)	1485056324
Grand Total	**1485056324**

Row Labels	Average of Calculated CFU/ml/OD:
S000554	**554293425.9**
Methylococcus capsulatus (Bath)	**554293425.9**
23	3444444444
50	916666666.7
55	618055555.6
65	9375000
70	99166.66667
75	0
80	0
90	0
100	0
Grand Total	**554293425.9**

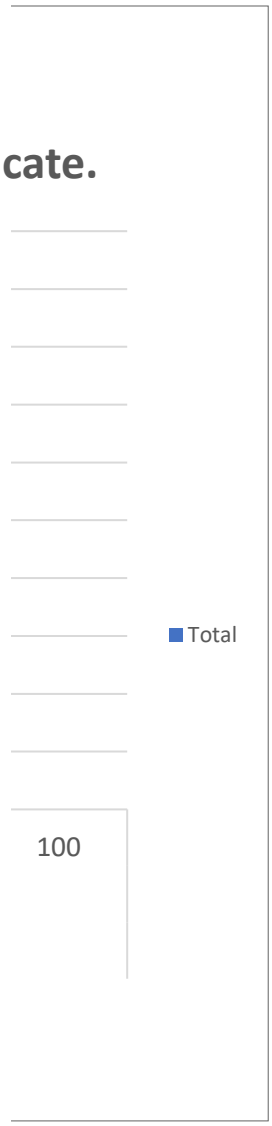
Row Labels	StdDev of Calculated CFU/ml/OD:
S000554	**1106015678**
Methylococcus capsulatus (Bath)	**1106015678**
23	637831250.2
50	85264091.37
55	79558605.82
65	3608439.182
70	10112.21758
75	0
80	0
90	0
100	0
Grand Total	**1106015678**

Average of Calculated CFU/ml/OD:



Strain#	Sample Description:	Heat Treatment Temperature (C):

***Samples were heated at the tested temperature for exactly 30 seconds.**



(b) (4)

Actual Number of Samples:	Replicate Number:	Sample Description:	Strain#	Targeted OD Normalized Sample:	Actual Final OD of Sample:	Volume of Heat Treated Sample (ml)	Heat Treatment Temperature (C):	Incubation Duration:	Plating Media:	Plating Dilution:	Volume Plated (ml):	Plating Method:	Plating Incubation Temperature (C):	Plating Incubation Time (Hrs):	CFU Count:	Calculated CFU/ml:	Calculated CFU/ml/OD:
1	1	Methylococcus capsulatus (Bath)	S000554	1		(b) (4)	23	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			(b) (4)
2	2	Methylococcus capsulatus (Bath)	S000554	1			23	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
3	3	Methylococcus capsulatus (Bath)	S000554	1			23	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
4	1	Methylococcus capsulatus (Bath)	S000554	1			50	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
5	2	Methylococcus capsulatus (Bath)	S000554	1			50	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
6	3	Methylococcus capsulatus (Bath)	S000554	1			50	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
7	1	Methylococcus capsulatus (Bath)	S000554	1			55	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
8	2	Methylococcus capsulatus (Bath)	S000554	1			55	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
9	3	Methylococcus capsulatus (Bath)	S000554	1			55	30 seconds	MMS2.0	1000000	0.1	Sterile Glass Beads	42	120			
10	1	Methylococcus capsulatus (Bath)	S000554	1			60	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
11	2	Methylococcus capsulatus (Bath)	S000554	1			60	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
12	3	Methylococcus capsulatus (Bath)	S000554	1			60	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
13	1	Methylococcus capsulatus (Bath)	S000554	1			65	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
14	2	Methylococcus capsulatus (Bath)	S000554	1			65	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
15	3	Methylococcus capsulatus (Bath)	S000554	1			65	30 seconds	MMS2.0	100000	0.1	Sterile Glass Beads	42	120			
16	1	Methylococcus capsulatus (Bath)	S000554	1			70	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
17	2	Methylococcus capsulatus (Bath)	S000554	1			70	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
18	3	Methylococcus capsulatus (Bath)	S000554	1			70	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
19	1	Methylococcus capsulatus (Bath)	S000554	1			75	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
20	2	Methylococcus capsulatus (Bath)	S000554	1			75	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
21	3	Methylococcus capsulatus (Bath)	S000554	1			75	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
22	1	Methylococcus capsulatus (Bath)	S000554	1			80	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
23	2	Methylococcus capsulatus (Bath)	S000554	1			80	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
24	3	Methylococcus capsulatus (Bath)	S000554	1			80	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
25	1	Methylococcus capsulatus (Bath)	S000554	1			90	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
26	2	Methylococcus capsulatus (Bath)	S000554	1			90	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
27	3	Methylococcus capsulatus (Bath)	S000554	1			90	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
28	1	Methylococcus capsulatus (Bath)	S000554	1			100	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
29	2	Methylococcus capsulatus (Bath)	S000554	1			100	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			
30	3	Methylococcus capsulatus (Bath)	S000554	1			100	30 seconds	MMS2.0	10	0.1	Sterile Glass Beads	42	120			

Cerrito, Chelsea

From: Pelonis, Evangelia C. <pelonis@khlaw.com>
Sent: Tuesday, January 10, 2023 10:03 AM
To: Conway, Charlotte
Cc: Mahoney, Jill M.; tbelloso@calysta.com; Animalfood-premarket
Subject: [EXTERNAL] RE: GRAS Notice No. AGRN 60 - Dried Methylococcus capsulatus product

Categories: Green Category

Dear Charlotte,

Thank you for taking the time to speak with me on Monday. We have spoken with Calysta and agree that "Dried Fermentation Biomass" is a reasonable common or usual name for Calysta's GRAS Notice for Dried *Methylococcus capsulatus* (*M. capsulatus*) product. Based on our discussion on Monday, we understand that the AAFCO Manual will list "Dried *Methylococcus capsulatus* Biomass" in the "Substance" column, and "Dried Fermentation Biomass" in the "Common or Usual Name" column at "Table 101.1. GRAS Notified Substances with No Questions Letters from the FDA". We also understand that "Dried Fermentation Biomass" will appear in FDA's response letter.

We appreciate the opportunity to discuss the appropriate common or usual name for Calysta's notified substance and we look forward to receiving CVM's response letter to AGRN 60.

Best,
Eve

Evangelia C. Pelonis
Partner

direct 202.434.4106 pelonis@khlaw.com
Keller and Heckman LLP | 1001 G Street NW, Suite 500 West | Washington, DC 20001

khlaw.com
dailyintakeblog.com

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From: Conway, Charlotte <Charlotte.Conway@fda.hhs.gov>
Sent: Friday, January 6, 2023 9:28 AM
To: Pelonis, Evangelia C. <pelonis@khlaw.com>
Cc: Mahoney, Jill M. <mahoneyj@khlaw.com>; tbelloso@calysta.com; Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>
Subject: GRAS Notice No. AGRN 60 - Dried Methylococcus capsulatus product

Good morning,

As we near completion of our evaluation of Calysta, Inc.'s GRAS notice for Dried *Methylococcus capsulatus* (*M. capsulatus*) product to be used as a source of protein in the diets of salmonid species, designated as GRAS Notice No. AGRN 60 that was filed on June 2, 2022, we have an outstanding question on the common or usual name. In your November 23, 2022 amendment, you proposed that the common or usual name be "Microbial protein from *Methylococcus.*" As we've considered the amendment, we have questions about this common or usual name. As we have considered this GRAS Notice, we looked at how similar substances are identified. Including AGRN 26, Dried *Methylobacterium extorquens* biomass, and AAFCO ingredient definition 36.15, Dried Fermentation Biomass. Both of these substances are similarly intended to be a source of protein in animal diets, produced by fermentation of microorganisms.

Although the name "Dried Fermentation Biomass" currently only applies to substances that meet the identity described in AAFCO Definition 36.15, this name also could be appropriate to describe the substance produced by the consortium of microorganisms described in your GRAS notice. Alternatively, we've considered that "Dried *Methylococcus capsulatus* Biomass" could be an appropriate name given the predominance of the *M. capsulatus* in the biomass. Please let us know at your earliest convenience if you agree that either of these names is acceptable. If we do not receive a response, we will proceed with our evaluation of the notice and identify the common or usual name in our letter.

Thank you,
Charlotte

Charlotte Conway, MS, PAS, Dpl. ACAS
Deputy Division Director

Center for Veterinary Medicine
Division of Animal Food Ingredients
U.S. Food and Drug Administration
Tel: 240-402-6768
charlotte.conway@fda.hhs.gov