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OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION

MEMORANDUM

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SUBJECT: **Paraquat Dichloride:** Systematic review of the literature to evaluate the relationship between paraquat dichloride exposure and Parkinson's disease

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1.0 Executive Summary

Paraquat dichloride is a restricted-use quaternary ammonium herbicide employed for weed control and as a harvest aid in the United States. It is currently undergoing Registration Review at the Office of Pesticide Programs (OPP). Respiratory toxicity is the most common effect associated with paraquat exposure. The impact of paraquat on the respiratory system is explored extensively in the open literature and is well-characterized along with toxicity in other target tissues (kidneys, ocular) and contact toxicity in the guideline studies submitted to OPP to meet data requirements for the pesticide registration process. The central nervous system has also received considerable attention in the paraquat literature with an emphasis on Parkinson's disease (PD) hallmarks including accumulation of α-synuclein in neurons (Lewy bodies), degeneration of vulnerable neuron populations including dopaminergic neurons in the midbrain, depletion of dopamine in the striatum, and impairment of motor and non-motor function. The OPP toxicity database does include several studies that explore general neurotoxicity and PD-specific hallmarks; however, OPP recognizes that these studies represent a small fraction of the available literature on neurotoxic outcomes related to paraquat exposure and PD.

As part of Registration Review, OPP conducted a fit-for-purpose systematic review to evaluate the significance and environmental relevance of the postulated association between paraquat exposure and PD. A literature database for the PD systematic review was compiled from three primary sources of data: the OPP paraquat toxicity database for registration, the OPP paraquat epidemiology review, and the National Toxicology Program (NTP) scoping review of open literature relevant to evaluating the association between paraquat exposure and PD. Data from the studies were separated into three lines of evidence – human, animal, and *in vitro* – and evaluated for quality, substance, and environmental relevance. Environmental relevance was defined as the likelihood that a given effect would result from an exposure scenario anticipated to occur from typical use of registered paraquat products (e.g. oral including dietary, dermal and inhalation exposure). OPP integrated environmental relevance considerations into the systematic review in order to contextualize hazard information in terms of risk. Studies that were of sufficient quality and investigated environmentally relevant exposure scenarios were then evaluated in their respective body of evidence and collectively across lines of evidence in the weight of evidence analysis.

A screen of the open literature and OPP toxicity database returned 28, 217, and 244 human, animal, and *in vitro* studies, respectively, that were relevant to evaluating the association between paraquat exposure and PD. Further review of the relevant animal open literature revealed that many of the studies used injection as the route of administration or were conducted with alternative mammalian models. OPP acknowledges that a number of injection studies report PD-like effects in rodents following exposure to paraquat; however, injection is not representative of the anticipated exposure scenarios for registered uses of paraquat due to differences in toxicokinetic behavior. These studies were thus excluded from consideration in the PD systematic review due to a lack of environmental relevance. Likewise, studies conducted with alternative mammalian models were excluded because they were determined to be of limited use to evaluating human health risk. Study evaluation of the *in vitro* database focused on the studies that reported the most sensitive response for relevant outcomes within the human and rodent models due to the density of relevant studies available. The *in vitro* studies excluded from study evaluation either presented results that were not meaningfully different from those reported in the evaluated studies, reported outcomes that were not relevant to the weight of evidence analysis, and/or the reported results indicated the *in vitro* model examined was not more sensitive than the relevant models discussed for a particular outcome. Additional studies from all three lines of evidence were excluded based on insufficient quality. In total, data from 26, 11, and 34 studies were considered in the evaluation

of the human, animal, and *in vitro* evidence, respectively, and integrated in the weight of evidence analysis. In addition, the 11 acceptable animal studies were considered in the selection of points of departure for the Registration Review risk assessment.

Evaluating the link between paraquat exposure and PD is reliant on the strength, consistency, and coherence of PD or PD-like hallmarks within and across the human, animal, and *in vitro* lines of evidence, and concordance with toxicokinetic and mechanistic data. Some evidence connecting environmentally relevant paraquat exposure to motor, neuropathological, and/or neurochemical hallmarks of PD was reported in the acceptable literature compiled for this systematic review; however, confidence in these positive findings was diminished by gaps in the dose and temporal concordance, mixed and conflicting results between and across lines of evidence, and unresolved uncertainties in the studies and overall weight of evidence.

The evaluation of 26 human studies considered were all epidemiologic articles and reported findings on 13 study populations, including three agricultural cohorts, nine hospital-based populations, and one PD registry in Nebraska. These study populations may have been exposed to paraquat through occupational and non-occupation exposure pathways that vary in terms of magnitude, frequency, and duration, with occupational study populations being more likely to experience exposure as a result of direct use of paraquat. With respect to occupational exposure, it was determined that there *is limited, but insufficient epidemiologic evidence of a clear associative or causal relationship.* This conclusion was based on mixed findings in both the Agricultural Health Study cohort and other study populations. These studies may all be subject to uncertainty due to limitations in their design, exposure assessment approach, and potential for bias. With respect to non-occupational study populations, evidence from three study populations was evaluated and it was determined that there is *insufficient epidemiologic evidence of a clear associative or causal relationship.* This conclusion was based on the small number of studies on non-occupational populations, lack of consistent evidence of a positive association, and the potential for bias.

Empirical evidence of motor impairment in laboratory animals was observed in male mice following oral exposure for at least 28 days to doses $\geq$7.2 mg ion/kg/day (10 mg dichloride/kg/day). These findings were the strongest evidence of neurotoxicity attributed to paraquat in the animal literature evaluated for this systematic review. The behavioral changes were observed across several studies that used a high purity paraquat product and exhibited a large magnitude of change from controls. Motor impairment was, however, not observed in female mice nor in rats of either sex. Only one animal study presented evidence to suggest the observed motor impairment in male mice was connected to dopaminergic neuron degeneration and neurochemical disruption – two hallmarks integral to the pathology of PD in humans – but there was not enough information in the study nor collectively in the animal literature to evaluate consistency, dose response, or temporal concordance. Toxicokinetic, *in vitro*, and mechanistic data added credibility to the positive findings in male mice but the lack of supporting empirical evidence for tissue, cellular, and biochemical PD-like hallmarks in the animal studies diminish confidence that the observed motor impairment was a result of a PD-like pathology in mice. Other environmentally relevant routes of exposure were less studied in the literature. No reliable evidence of PD-like hallmarks was observed in mice or rats after repeated intranasal exposure, which was consistent with the toxicokinetic data indicating paraquat did not distribute to the ventral midbrain or striatum after acute exposure. No data were available to evaluate PD-like hallmarks following dermal exposure; however, systemic paraquat concentration is expected to be low following dermal exposure provided the dermal dose does not reach levels that affect the integrity of the skin. Overall, the limited, mixed findings in the animal literature were considered weak evidence of a PD-like response to paraquat exposure.

Qualitative similarities in the positive findings for *in vitro* and behavioral outcomes between rodents and humans indicated some interspecies coherence in the neurological response to paraquat exposure; however, there was a lack of coherence for tissue, cellular, subcellular, and biochemical PD hallmarks, in part because few animal studies and no human studies investigated these hallmarks. The small number of positive findings and the lack of consistency in the findings in the human studies also diminished confidence in the biological plausibility of the animal and *in vitro* findings. Occupational and dietary exposure in humans resulting from pesticidal use of paraquat products currently registered in the United States is not estimated to reach external dose levels that elicited PD-like effects in whole animal studies. These estimates may not apply for uses outside of the United States but do suggest that the PD-like outcomes observed in the laboratory are not likely to occur from label-directed use in the US. Given the weakness within and across lines of evidence and the exposure considerations outlined above, OPP concluded that the weight of evidence was insufficient to link paraquat exposure from pesticidal use of US registered products to PD in humans. OPP did not evaluate the adverse outcome pathways (AOP) proposed in the open literature nor develop one from the data gathered in the systematic review. Given the lack of sufficient evidence for a causal association, OPP did not consider an AOP necessary to characterize paraquat toxicity and evaluate risk for registered products.

The findings of this systematic review were integrated with the rest of the paraquat toxicity profile in the hazard characterization and were considered in the point of departure selection and uncertainty factor determination for the Registration Review human health risk assessment. In selecting the most sensitive point of departure to estimate risk, the Registration Review risk assessment accounted for all forms of treatment-related adversity reported for paraquat including the neurotoxic effects discussed in this systematic review. The toxicity profile for paraquat indicates that contact toxicity and effects in the respiratory and renal system occur at lower doses than those eliciting neurotoxicity in animal models. Paraquat is also lethal to pregnant rats at the doses reported to elicit neurotoxicity. Based on these findings, it is expected that a multitude of contact and systemic effects would precede the PD-like neurotoxic effects reported in the literature. Contact, renal, and respiratory toxicity are, therefore, of greater concern to human health and more relevant to assessing risk from paraquat exposure during routine use of pesticidal products with US registration. Points of departure selected for risk assessment were thus based on the more sensitive respiratory effects and are protective of the neurotoxic effects attributed to paraquat exposure discussed in this systematic review.

2.0 Introduction

Paraquat dichloride (hereafter referred to as paraquat) is a restricted-use quaternary ammonium herbicide employed for weed control and as a harvest aid in the United States. Its pesticidal mode of action – generation of reactive oxygen species (ROS) in chloroplasts leading to ubiquitous oxidative stress and eventual cell death (Hawkes 2014) – is highly effective for combating weed pressure in agricultural settings. Toxicity in mammals manifests through a similar mode of action: formation of ROS at the cellular level incurs oxidative stress in tissues that progresses to severe tissue injury or death within a narrow dose range (Dinis-Oliveira et al. 2008). Tissue damage can occur locally at the point of contact as well as systemically. At the point of contact, paraquat corrodes protective barriers that otherwise effectively limit absorption into systemic circulation. Upon absorption into systemic circulation, paraquat primarily targets the respiratory system. Respiratory toxicity is explored extensively in the open literature and is well-characterized along with toxicity in other target tissues (kidneys, ocular) and contact toxicity in United States Environmental Protection Agency (EPA) Office of Chemical Safety and Pollution Prevention (OCSPP) guideline toxicity studies submitted by the paraquat registrants to the EPA Office of Pesticide Programs (OPP).

The central nervous system has also received considerable attention in the paraquat literature with a particular emphasis on exploring a hypothesized link between exposure to paraquat and Parkinson's disease (Baltazar et al. 2014). Parkinson's disease (PD) is a progressive neurodegenerative disease that is associated with motor and non-motor behavioral changes in affected humans (Anthony et al. 2013). The pathology of PD is characterized by accumulation of α-synuclein in neurons (Lewy bodies), degeneration of vulnerable neuron populations including dopaminergic neurons in the midbrain, and depletion of dopamine in the striatum. The OPP toxicity database for paraquat is comprised of six neurotoxicity studies – two guideline neurotoxicity studies (acute and subchronic neurotoxicity battery) and four non-guideline studies designed to examine outcomes related to PD following dietary exposure or intraperitoneal injection. These studies report minimal evidence of neurotoxicity following oral exposure to paraquat; however, it was recognized that they represent a small fraction of the available literature on neurotoxic outcomes related to paraquat exposure. As part of the Registration Review process for paraquat, OPP conducted a fit-for-purpose systematic review to evaluate the association between paraquat exposure and PD (hereafter referred to as the PD systematic review).

Prior to the start of the systematic review, OPP reached out to the National Toxicology Program (NTP) for their expertise in conducting systematic reviews. NTP had previously identified the association between paraquat and PD as a potential candidate for review and offered to collaborate on the project. With input from OPP, NTP conducted a scoping review of the open literature to identify and summarize the studies that were relevant to evaluating the paraquat-PD association. The resulting open literature database compiled for the scoping review served as one of the three main data resources for the OPP systematic review. In addition to the collaboration on the scoping review, experts at NTP also provided technical assistance on the systematic review process and addressed questions pertaining to neuropathology and PD that aided interpretation of study results. The NTP experts were not involved in the initial data evaluation and weight of evidence analysis; however, the experts reviewed the systematic review memo prior to publication and OPP incorporated their feedback into these sections.

The objective of the PD systematic review was to evaluate the significance and environmental relevance of the postulated association between paraquat exposure and PD by integrating evidence from the paraquat OPP toxicity database and open literature human, animal, and *in vitro* studies. Environmental relevance was defined as the likelihood that a given effect would result from an exposure scenario anticipated to occur from typical use of registered paraquat products (e.g. oral including dietary, dermal and inhalation exposure). OPP integrated environmental relevance considerations into the systematic review in order to contextualize hazard information in terms of risk. The PD systematic review was designed to accomplish the following:

- Identify studies from OPP database and open literature reporting neurobehavioral, neurochemical, and neuropathological endpoints associated with PD in humans, animals, or *in vitro* model systems that can be linked to anticipated exposure scenarios (e.g. oral including dietary, dermal, or inhalation) for registered paraquat products.
- Evaluate the quality of human, animal, and *in vitro* studies
- Synthesize the evidence from the open literature and relevant toxicity studies in the OPP toxicity database using a narrative approach.

The conclusions of the weight of evidence analysis of the human, animal, and *in vitro* data were incorporated into the broader discussion of human health effects from paraquat exposure in the Registration Review human health risk assessment (D430827, W. Britton, 06/26/2019) and will be used to inform risk management decisions for active paraquat registrations.

3.0 Document Overview

In recent years, the National Academy of Sciences National Research Council (NRC) has encouraged the agency to move towards systematic review processes to enhance the transparency of scientific literature reviews that support chemical-specific risk assessments to inform regulatory decision making (NRC 2011). The NRC defines systematic review as "a scientific investigation that focuses on a specific question and uses explicit, pre-specified scientific methods to identify, select, assess, and summarize findings of similar but separate studies" (NRC 2014). Consistent with the NRC's recommendations, OCSPP is currently developing systematic review policies and procedures. In short, OCSPP employs "fit for purpose" systematic reviews that rely on standard methods for collecting, evaluating, and integrating the scientific data supporting the agency's decision. The concept of fit for purpose implies that a particular activity or method is suitable for its intended use. Inherent in this definition is the concept that one size does not fit all situations and thus flexibility is allowed. However, it is notable that with flexibility comes the importance of transparency of documented process including approaches to data collection, evaluation, and integration. Accordingly, this document provides a transparent account of the fit for purpose systematic review process used by OPP to evaluate the association between paraquat exposure and PD. The document is organized as follows:

- *Section 4.0 Compiling the PD Systematic Review Literature Database* provides information on the three primary data sources used to compile the literature database for this systematic review including details on methods used to screen the open literature and the results of the open literature screen
- *Section 5.0 Human Data Evaluation* presents the results of the human study quality assessment and a comprehensive evaluation of relevant human data
- *Section 6.0 Animal Data Evaluation* presents the results of the animal study quality assessment and a comprehensive evaluation of relevant animal data
- *Section 7.0 In vitro Data Evaluation* presents summaries of the relevant *in vitro* studies from the PD systematic review literature database and a comprehensive evaluation of relevant *in vitro* data
- *Section 8.0 Weight of Evidence Analysis* integrates relevant human, animal, and *in vitro* data into a weight of evidence analysis to evaluate the association between environmentally relevant paraquat exposure and PD.
- *Section 9.0 Implications for Registration Review Risk Assessment* discusses the impact of the findings and conclusions of the PD systematic review on the Registration Review risk assessment.
- *Section 10.0 Conclusions*
- *Section 11.0 References*
- *Appendix 1* includes detailed information on the search strategy, screening inclusion/exclusion criteria, and study quality evaluation criteria for the epidemiology systematic review
- *Appendix 2* includes detailed information on the search strategy, screening inclusion/exclusion criteria, and binning criteria for the NTP scoping review
- *Appendix 3* details communications with the NTP neuropathology and PD experts

4.0 Compiling the PD Systematic Review Literature Database

A comprehensive and complete collection of relevant literature is of critical importance to evaluate associations between chemical exposure and health outcomes. The literature database for the PD systematic review was compiled from three primary sources of data: the OPP paraquat toxicity database for registration (Section 4.1), the OPP paraquat epidemiology review (Section 4.2), and the NTP scoping

review of open literature relevant to evaluating the association between paraquat exposure and PD (Section 4.3).

4.1 OPP Toxicity Database

The OPP toxicity database consists of unpublished guideline and non-guideline studies submitted to the agency voluntarily or to fulfil the 40 CFR Part 158 toxicology data requirements for pesticide registration. These studies cover multiple durations of exposure (acute, subchronic, and chronic) and report toxicity in several mammalian species (rodents, rabbits, and dogs). The outcomes reported in this database are varied and are discussed in detail in the paraquat Registration Review risk assessment (D430827 W. Britton 2019). Although clinical observations reported in each study provide some insight into the general health of the animals, the most relevant information for this systematic review was contributed by the studies that were designed to identify specific neurotoxic outcomes, including hallmarks of PD. Relevant studies in the toxicity database included two OCSPP guideline neurotoxicity studies (acute and subchronic neurotoxicity battery) and four non-guideline studies designed to examine outcomes related to PD following dietary exposure or intraperitoneal injection.

4.2 OPP Epidemiology Review

As part of the paraquat Registration Review, OPP conducted a review of the epidemiology literature on the association between paraquat exposure and adverse human health outcomes. The epidemiology review process, including methods used to screen the epidemiology literature, compile the epidemiology literature database, and assesses study quality, follows the OPP *Framework for Incorporating Human Epidemiologic & Incident Data in Risk Assessments for Pesticides* [1] (herein called the OPP Epidemiology Framework) and is further described in the OPP paraquat dichloride epidemiology report (D449108, A. Niman, 06/26/2019). The screening and evaluation methods described in the epidemiology report are also summarized here (supplemental information is included in Appendix A.1) to explain the origins of the epidemiology literature referenced in the PD systematic review and to aid interpretation of the epidemiology data discussed throughout this document.

4.2.1 Epidemiology Literature Screen Methods

The paraquat epidemiology review followed the procedures outlined in the OPP Epidemiology Framework and examined PD and other adverse health outcomes. The literature search strategy was designed based on population, exposure, comparator, and outcome of interest (PECO) criteria developed exclusively for the epidemiology review:

- **Population of interest:** Population studied must be humans with no restrictions, including no restrictions on age, life stage, sex, country of residence/origin, race/ethnicity, lifestyle, or occupation
- **Exposure:** Exposure studied must be to paraquat in any application via any route of exposure.
- **Comparator:** Exposed or case populations must be compared to a population with low/no exposure or to non-cases to arrive at a risk/effect size estimate of a health outcome associated with paraquat exposure.
- **Outcome:** All reported human health effects, with no restrictions on human system affected (effects could be based on survey or other self-report, medical records, biomarkers, publicly available health data, or measurements from human sample populations).

[1] US EPA. December 28, 2016. Office of Pesticide Programs' Framework for Incorporating Human Epidemiologic & Incident Data in Risk Assessments for Pesticides. https://www3.epa.gov/pesticides/EPA-HQ-OPP-2008-0316-DRAFT-0075.pdf

Search terms were selected based on the PECO criteria and included specific keywords for paraquat such as chemical name and synonyms, general terms for health effects/disease, exposure, and methods commonly found in epidemiology studies, and exclusion terms for animal models (Appendix A.1.1). The search was conducted in the PubMed, PubMed Central, Science Direct, Toxline, SCIELO (Scientific Electronic Library Online), and SciSearch publication databases and was not restricted by publication date. A supplemental search of publications resulting from the Agricultural Health Study (AHS) and a review of reference article citation lists were conducted to identify studies that were not captured in the open literature database searches. Citations identified in the searches were screened for relevance using inclusion/exclusion criteria (Appendix A.1.2) developed from the epidemiology PECO criteria.

4.2.2 Epidemiology Literature Screen Results

Five hundred and seventy-six (576) unique articles were identified across the search engines. The title and abstract of each article was screened for potential relevance using the inclusion/exclusion criteria described in Appendix A.1.2. After screening articles, reviewing reference article citations, and performing a supplemental search of AHS publications, a total of 74 peer-reviewed epidemiological articles that were relevant to the epidemiology systematic review. The broad search strategy was designed to capture all possible outcomes reported in the epidemiology literature and the resulting epidemiology database included studies that explored associations with PD, lung and respiratory effects, cancer, and a number of other outcomes. Of the 74 relevant articles, 26 investigated the association between paraquat exposure and PD and were considered in the PD systematic review.

4.3 NTP Scoping Review

NTP, with input from OPP, conducted a scoping review of the paraquat open literature to identify relevant publications reporting information on PD in humans, and animal and *in vitro* models. The purpose of the scoping review was to summarize and evaluate the information presented in relevant publications and identify limitations and areas of research that need to be investigated further. The scoping review was not intended to evaluate study quality or analyze the weight of evidence for the PD-paraquat association. The search strategy, screening methods, and inclusion/exclusion criteria developed by NTP are summarized here and are reported in more detail in the NTP scoping review protocol (NTP 2018)[2].

4.3.1 NTP Scoping Review Literature Screen Methods

The search strategy employed in the NTP scoping review cast a wide net to ensure that a significant portion of the literature on paraquat exposure and PD was considered in the evaluation. The literature search strategy was designed based on the PECO criteria established for each evidence stream (human, animal, *in vitro*):

Human

- **Population of interest:** Humans, without restriction on age, sex, or lifestage at exposure or outcome assessment.
- **Exposure:** Exposure to paraquat dichloride (CAS#1910-42-5) based on administered dose or concentration, biomonitoring data (e.g., urine, blood, or other specimens), environmental measures (e.g., air, water levels), or indirect measures such as job title or occupational history.
- **Comparator:** A comparison population exposed to lower levels (or no exposure/exposure below detection levels) of paraquat.

[2] https://ntp.niehs.nih.gov/ntp/ohat/parkinson/parkinsons_protocol_508.pdf

- **Outcome:**
    - **Primary outcomes:** Diagnosis of Parkinson's disease and/or clinical observations, neurobehavioral, or neuropathological outcomes typically associated with Parkinson's disease or parkinsonism following *in vivo* exposure, focusing on tissue level and functional abnormalities, descriptive and/or functional assessment of the central nervous system, including the nigrostriatal (dopamine) system. Examples of relevant outcomes include tremor, bradykinesia, rigidity, postural instability, and any other movement abnormalities associated with parkinsonism.
    - **Secondary outcomes:** Tissue, cellular, biochemical, and/or molecular outcomes resulting from *in vivo* exposure that have a mechanistic association with Parkinson's disease or are evidence of toxicity in the nervous system but are not specific to Parkinson's disease.

Animal

- **Population of interest:** Experimental animals without restriction on species (including non-mammalian and invertebrate species), age, sex, or lifestage at exposure or outcome assessment.
- **Exposure:** Exposure to paraquat dichloride (CAS#1910-42-5) based on administered dose or concentration.
- **Comparator:** Comparable animal populations that were untreated or exposed to vehicle-only treatment in experimental animal studies.
  Outcome:
    - **Primary outcomes:** Neurobehavioral or neuropathological outcomes, focusing on whole body and tissue level abnormalities typically associated with Parkinson's disease following *in vivo* exposure. Endpoints include motor activity and coordination, sensorimotor reflexes, effects on cognitive function, quantitative or qualitative assessment of dopaminergic neuron counts in the substantia nigra and dopaminergic neuron terminals in the striatum, and other descriptive and/or functional assessments of the central nervous system including the nigrostriatal (dopamine) system that are considered hallmarks of Parkinson's disease (e.g., detection of intracytoplasmic Lewy bodies).
    - **Secondary outcomes:** Tissue, cellular, biochemical, and/or molecular outcomes resulting from *in vivo* exposure that have a mechanistic association with Parkinson's disease (e.g. dopamine and metabolite levels in the nigrostriatal pathway, TH+ immunoreactivity density) or are evidence of toxicity in the nervous system, but are not specific to Parkinson's disease (e.g. oxidative stress, inflammation, mitochondrial and/or proteasomal dysfunction)

In vitro

- **Population of interest:** Human or animal cells, tissues, or model systems with *in vitro* exposure regimens. Examples of cell lines typically used for *in vitro* Parkinson's disease mechanistic study include: SK-N-SH, SH-SY5Y, PC12, RBE, astrocytes, and dopaminergic neurons.
- **Exposure:** Exposure to paraquat dichloride (CAS#1910-42-5) based on administered dose or concentration.
- **Comparator:** Comparable cells or tissues exposed to vehicle-only treatment or untreated controls.
- **Outcome:** *In vitro* assays investigating cellular responses commonly attributed to Parkinson's disease (e.g., assessment of functionality, integrity, and viability for nerve cells critical to the

nigrostriatal [dopamine] system) and mechanistic assays investigating proposed pathways for the etiology of Parkinson's disease (e.g., enzyme interactions, cell signaling).

Searches of peer-reviewed published literature were conducted in EMBASE, PubMed, Scopus, Web of Science, and Toxline without publication year restriction using key terms specific to paraquat such as chemical name and synonyms, descriptive terms for the hallmarks of PD, and general terms for neurotoxicity. The search terms used for each search engine as well as the dates and times of the searches are presented in the NTP scoping review protocol (NTP 2018) and Appendix A.2.1. NTP performed the title/abstract and full text screen of the citations returned from each database using inclusion/exclusion criteria developed based on the PECO criteria for each evidence stream. The inclusion/exclusion criteria are outlined in the NTP scoping review protocol (NTP 2018) and reproduced in Appendix A.2.2. After screening the open literature databases, NTP hand searched all relevant published reviews for articles that were not captured and screened them for relevance based on the same inclusion/exclusion criteria. Studies that satisfied the criteria were binned based on the evidence stream and the outcomes reported. The NTP scoping review distinguished between primary and secondary outcomes for the human and animal evidence streams for the purposes of data extraction. *In vitro* data were considered supporting information for the other evidence streams; therefore, the NTP scoping review did not distinguish between primary and secondary outcomes for *in vitro* studies. In addition to binning studies, NTP extracted relevant information on study characteristics to support study evaluation including model species and strain, routes and levels of exposure, and outcomes assessed.

4.3.2 NTP Scoping Review Literature Screen Results

The search strategy (database searches plus hand screening of reference lists in review papers) returned 7,166 unique articles across the three evidence streams. At the title/abstract screening level, 6,152 studies were excluded because they were not relevant based on the PECO criteria and 120 citations were identified as review articles. Full text screening excluded 426 of the remaining 894 articles because they did not satisfy the PECO criteria and identified an additional 10 review articles. In total, the NTP scoping review identified 458 studies that were relevant for evaluating the association between paraquat exposure and PD. Of these 458 studies, 25 contained information on human outcomes (24 primary and 1 secondary), 214 for animal outcomes (143 primary and 190 secondary), and 244 for *in vitro* outcomes. Some of the studies contained information for multiple evidence streams and/or both primary and secondary outcomes, which explains why the citation numbers for each evidence stream and outcome category do not add up to the total number of relevant studies. A full citation list of the relevant human, animal, and *in vitro* studies identified by NTP in the title/abstract and full text screen is provided in the NTP scoping review memo (NTP 2019, in press).

5.0 Human Data Evaluation

5.1 Study Evaluation Methods

All human studies considered for the PD systematic review were epidemiology investigations. Accordingly, the OPP evaluation adhered to the OPP Epidemiology Framework. [3] Relevant information from the human studies was summarized on study design, results, conclusions, and the strengths and weaknesses of each study per the OPP Epidemiology Framework, and recount details including the exposure measurement, outcome ascertainment, number of participants (n), number exposed/number of cases, number in reference (un-exposed/control) group, effect measure (e.g., odds ratio (OR), relative risk

[3] US EPA. December 28, 2016. Office of Pesticide Programs' Framework for Incorporating Human Epidemiologic & Incident Data in Risk Assessments for Pesticides. https://www3.epa.gov/pesticides/EPA-HQ-OPP-2008-0316-DRAFT-0075.pdf

(RR), hazard ratio (HR)) and associated estimate of uncertainty and/or statistical significance (e.g., confidence interval (CI), p-value), confounders considered, and methods of analysis. OPP considered these elements in assessing the quality of each publication and its applicability to an overall assessment of the health effects associated with paraquat exposure.

Study quality was evaluated based on the epidemiology study quality considerations outlined in the OPP Epidemiology Framework and in Appendix A.1.3. The study quality assessment considered aspects such as design, conduct, analysis, and interpretation of study results, including whether study publications incorporated a clearly articulated hypothesis, adequate assessment of exposure, critical health windows, valid and reliable outcome ascertainment, a sample representative of the target population, analysis of potential confounders, characterization of potential systematic biases, evaluation and reporting of statistical power, and use of appropriate statistical modeling techniques.

Study design influenced the assessment of study quality. Cohort studies, which enable researchers to assess the temporality of exposure in relation to health outcome and to consider multiple health outcomes, were generally considered higher quality than other study designs. Case-control studies, which are susceptible to recall bias, were generally considered lower quality than nested case-control studies, which may be less susceptible to selection and recall bias.[4] Cross sectional studies cannot distinguish temporality for exposure in relation to health outcomes; therefore, cross-sectional studies were generally considered lower quality than cohort or case-control studies, and were regarded as hypothesis-generating in the absence of additional studies supporting an observed association. The lowest quality study design considered was ecologic studies, due to an inability to extrapolate observed associations from the group level to the individual level (ecological fallacy) inherent in the ecologic study design. Ecologic studies were generally regarded as hypothesis-generating studies.

Studies that characterized the exposure-response relationship (e.g., with a dose-response curve or trend statistic) were, in general, considered higher quality than studies that did not characterize exposure-response. Studies that specified temporality (i.e., those that determined exposure preceded a health outcome) and studies that specified uncertainties in the analysis were, in general, considered higher quality than studies that failed to specify temporality and studies that lacked an examination of uncertainty. Consistent results between study groups (e.g., a significant and positive association seen for both farmers and commercial applicator study groups within a single study) bolstered the assessment of study quality.

Risk estimates (estimates of effect) reported in epidemiological studies were generally considered as follows:

- no evidence of a positive association between exposure and outcome (e.g., OR $\leq$ 1.00);
- no evidence of a significant positive association (e.g., OR > 1.00 but not significant);
- evidence of a slight positive association (e.g., 1.00 < OR < 1.30 and significant);
- evidence of a positive association (e.g., 1.30 $\leq$ OR < 2.0 and significant);
- evidence of a moderately strong (e.g., 2.0 $\leq$ OR < 3.0 and significant) or strong (e.g., OR $\geq$ 3.0 and significant) positive association.[5]

[4] US EPA. December 28, 2016.

[5] For articles that reported ORs, RRs, and HRs, the confidence interval (CI) acted as a proxy for significance testing, with CIs that do not contain the null value (OR / RR / HR = 1.00) considered significant. P-value significance considered a critical value of $\alpha = 0.05$ unless otherwise specified by the authors and noted in the summaries here.

However, we recognize that results that fail to attain statistical significance may still indicate clinical, biological, and/or public health importance and may warrant further exploration.[6] We particularly noted large observed associations (e.g., OR ≥ ~2.5) even in the absence of significance, perhaps indicating a smaller than optimal sample size. Conversely, we also recognized that statistical significance does not necessarily imply clinical or biological importance, particularly with larger than necessary sample sizes and other study elements that influence the reliability of estimated effects.

5.2 Results of Study Quality Evaluation

Combined and with duplicates removed, the NTP scoping review and OPP epidemiology review identified 28 human studies as relevant for evaluating the association between paraquat exposure and PD. There were no relevant human studies in the OPP toxicity database. Although the citation lists were generally in agreement, the NTP scoping review identified 2 studies that were not included in the 26 PD studies assessed in the OPP epidemiology report. The OPP report reviewed the Tomenson and Campbell (2011) and the Ranjabar *et al.* (2002) studies; however, they were not included in the PD weight of evidence discussion because the outcomes (e.g. mortality and oxidative stress) were not specific to PD. These two studies were also excluded from the PD systematic review. In total, 26 human studies were evaluated for the PD systematic review.

The relationship between paraquat exposure and Parkinson's disease (PD) was evaluated in 13 study populations, including three agricultural cohorts, nine hospital-based populations, and one PD registry in Nebraska. For several populations, the results on the relationship between paraquat and PD are described in multiple articles, typically a primary article that specifically examined the association between paraquat and other pesticides with PD and (potentially multiple) secondary articles that subsequently examined potential effect modification by environmental, dietary, and behavioral factors. The study participants (*e.g.,* cases and controls) included in these secondary articles overlap with the participants in the primary articles; therefore, these secondary articles help further characterize and extend the findings of primary articles but do not provide additional, independent information on any putative association between paraquat and PD. The results of OPP's study quality assessment for each of the 26 epidemiology studies investigating PD are summarized below and in the OPP paraquat dichloride epidemiology report (D449108 A. Niman 2019).

5.2.1 Agricultural-Based Study Populations

The PD systematic review literature database consisted of 8 studies on agricultural-based study populations. Six studies were conducted within the AHS cohort, one study was conducted within the French Agriculture and Cancer (AGRICAN) cohort, and one study was based on a cohort documented previously by the Washington State Department of Health.

*Agricultural Health Study (AHS) and Farming and Agricultural Movement Evaluation (FAME) Study (**High Quality**: FAME Study [Goldman et al., 2012; Furlong et al., 2015; Tanner et al., 2011; Kamel et al., 2014]; **Moderate Quality**: Kamel et al., 2007; Shrestha et al., 2018)*

AHS is a large cohort study that began enrollment in 1993. Potential AHS participants were identified from among individuals applying for certification to use restricted-use pesticides in Iowa and North Carolina. AHS originally enrolled 52,393 private applicators, 32,345 spouses, and 4,916 commercial applicators. Follow-up of the AHS cohort by collecting information using Phase 2, Phase 3, and Phase 4

[6] US EPA. December 28, 2016.

interviews in 1999-2003, 2005-2010, and 2013-2015, respectively, to evaluate cancer and non-cancer endpoints, including Parkinson's disease. Commercial applicators participated in Phase 2 follow-up but did not participate in subsequent phases of follow-up in AHS.[7]

Numerous add-on studies of specific health outcomes have leveraged the AHS study cohort to evaluate specific health outcomes in more detail. FAME is an AHS add-on study that used a case-control design nested within the AHS cohort to evaluate potential risk factors for PD. Using the AHS cohort, subjects suspected to have PD based on diagnoses from self-reports or state mortality files were screened cases. Screened controls were selected by stratified random sampling of all AHS participants. Controls were frequency-matched to cases by age (< 40, 40–49, 50–59, 60–64, 65–69, ≥ 70 years), sex, and state (Iowa or North Carolina) at a ratio of approximately three controls per case. The FAME study screened 170 cases and 644 controls. After screening cases and controls, the FAME study enrolled 115 cases and 383 controls after accounting for ineligible subjects, refusals, etc.

Five articles examined the relationship between paraquat exposure and PD within the AHS cohort, including one study of the entire AHS cohort (Kamel et al., 2007) and four FAME studies that relied on many of the same PD cases and used a case-control design to assess relationship between paraquat and PD (Tanner et al., 2011) and effect modifiers; these effect modifiers investigated included gene-by-environment interaction (Goldman et al., 2012), dietary fat intake (Kamel et al., 2014), and behavioral factors related to reducing occupational pesticide exposure (Furlong et al., 2015). In addition to these five articles, a more recent AHS publication reported on the association between paraquat use, as well as use of other pesticides, and the prodromal PD symptom dream enacting behavior (Shrestha et al., 2018).

The results of the five PD studies and one study on dream enacting behavior are summarized below:

Examination of Self-Reported PD in AHS Cohort

- Kamel et al. (2007) investigated the relationship between self-reported pesticide exposure in the AHS cohort and prevalent PD cases identified at enrollment (1993-1997) and incident PD cases identified during Phase 2 follow-up (1999-2003). At enrollment, study subjects, including pesticide applicators and their spouses, provided detailed information on lifetime pesticide use. Enrollment and follow-up questionnaires were also used to determine whether subjects reported a physician-diagnosed PD. There were 83 study subjects reporting PD diagnosis at enrollment (prevalent cases) and 78 study subjects reporting PD diagnosis after AHS (incident cases). Logistic regression was used to evaluate the relationship of either prevalent PD or incident PD to general pesticide use and specific pesticides, including paraquat, adjusting for age, state, and type of participant (applicator or spouse), race, education, and smoking. Based on this approach, the investigators reported no evidence of a significant positive association with prevalent PD (OR = 1.8; 95% CI, 1.0-3.4, n = 14 paraquat exposed cases) and no evidence of an association with incident PD (OR = 1.0; 95% CI, 0.5-1.9, n = 11 paraquat exposed cases).[8]

Examination of Clinically Confirmed PD in FAME Nested Case-Control Study of AHS Cohort

- Tanner et al. (2011) investigated the relationship between pesticides, including paraquat, in the FAME nested case-control study. The FAME study included 115 cases and 383 frequency-matched controls, of which 110 cases and 358 controls provided complete information available on pesticide use and application practices. Computer-assisted telephone interviews were used to

[7]AHS Website. About the Study: Available online at: https://aghealth.nih.gov/about/.

[8] Epidemiologist distinguish between incident and prevalent case of disease when quantifying the disease rate in a population. *Incident Cases* are new cases of disease in a population of interest and *Prevalent Cases* are existing cases of disease in a population of interest (Rothman and Greenland, 1998). In the context of AHS, incident PD cases reported diagnosis of PD after enrollment in AHS, whereas prevalent PD cases reported PD diagnosis during enrollment.

obtain detailed information on pesticide use for 31 selected pesticides. For every subject, each pesticide was categorized by ever/never use and lifetime days of use. Of the 31 pesticides selected, 18 pesticides were reported to be used by at least 10 subjects and individually analyzed using logistic regression. Based on this approach, the investigators reported evidence of a moderately strong positive association for ever use of paraquat (OR = 2.5, 95% CI, 1.4-4.7, n = 23 exposed cases). The investigators further examined the cumulative lifetimes days of paraquat exposure and reported that the effect estimate increased from an OR of 2.4 (95% CI: 1.0-5.5, n = 10 exposed cases) in individuals reporting ≤ median paraquat use of 8 lifetime days to an OR of 3.6 (95% CI: 1.6-8.1, n = 13 exposed cases) in individuals reporting > median paraquat use of 8 lifetime days.

- In additional analysis of the FAME case-control study, Goldman et al. (2012) investigated whether the risk of Parkinson's disease associated with paraquat exposure is modified by polymorphisms in the genes encoding for glutathione S-transferase M1 (GSTM1) and glutathione S-transferase T1 (GSTT1). The investigators genotyped 87 cases of Parkinson's disease and 343 controls matched on age, gender, and state of residence. Exposure to paraquat was either self-reported or reported by a proxy respondent and, for the interaction analysis, characterized as either "ever" versus "never" exposed. Years of lifetime paraquat use was also assessed and stratified into three categories: (i) never used; (ii) used less than the median of 4 years; or (iii) used more than the median. Unconditional logistic regression was used to estimate unadjusted and covariate-adjusted (age, sex, state, and cigarette smoking) odds ratios for self-reported paraquat exposure and Parkinson's disease and for the evaluation of multiplicative paraquat exposure effect modification by polymorphisms in the genes encoding GSTM1 and T1 GSTT1. As previously reported by Tanner et al. (2011), the investigators reported evidence of moderately strong positive association between paraquat use and PD (OR = 2.6, 95% CI: 1.3-5.0, n = 21 exposed cases). With regard to interaction between GSTT1 and paraquat use, there was no evidence of a significant positive association among paraquat users with functional GSTT1 (OR = 1.5, 95% CI 0.6-3.6, n = 12 exposed cases with functional GSTT1) relative to non-exposed male participants with functional GSTT1. However, paraquat users with the homozygous deletion of GSTT1 had an 11-fold increased odd of Parkinson's disease, relative to non-exposed male participants with homozygous deletion of GSTT1 (OR = 11.1; 95% CI: 3.0-44.6, n = 9 exposed cases). A similar interaction between paraquat exposure and GSTM1 genotype was not observed (data not reported in manuscript). Based on this analysis of interaction between GSTT1 and paraquat use, the investigators reported evidence that the GSTT1 genotype was a statistically significant modifier of the relative odds of Parkinson's disease comparing paraquat-exposed and non-exposed study participants (p-interaction: 0.027).
- In additional analysis of the FAME case-control study, Kamel et al. (2014) investigated if the potential association of PD with paraquat or rotenone is modified by dietary fat intake. Food intake was assessed using a self-administered food frequency questionnaire. Total energy and dietary fats were estimated using Diet*Calc software version 1.4.3. Daily intakes of total fat, saturated fat, monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), and individual PUFAs were expressed as a percentage of total energy (nutrient density), and then categorized in tertiles based on distributions in the control group; the lowest tertile was used as the reference group. Multivariable logistic regression models were used to perform the analyses, adjusting for age, sex, state, smoking, and total energy. The analyses for paraquat included 61 cases (18 exposed to paraquat) and 239 frequency-matched male controls (46 exposed to paraquat). With regard to paraquat and potential effect modification with dietary fat, the OR for paraquat was 4.2 (95% CI 1.5-11.6, n = 11 paraquat exposed cases) in individuals with low PUFA intake but 1.2 (95% CI 0.4-3.4, n = 7 exposed to paraquat) in those with high PUFA intake (p-interaction=0.10). The OR for paraquat was 4.0 (95% CI 1.5-10.9, n = 11 paraquat exposed cases) in individuals with low N-6 (omega 6) PUFA intake but 1.2 (95% CI 0.4-3.3, n = 7

exposed to paraquat) in those with high N-6 (omega 6) PUFA intake (p-interaction=0.08). The OR for paraquat was 3.8 (95% CI 1.4-10.3, n = 11 paraquat exposed cases) in individuals with low linoleic acid intake but 1.2 (95% CI 0.4-3.3, n = 7 paraquat exposed cases) in those with high linoleic acid intake (p-interaction=0.09).

- In additional analysis of the FAME case-control study, Furlong et al. (2015) investigated whether use of gloves and workplace hygiene modified the association between pesticide exposure and PD. The investigators collected questionnaire data on the use of protective gloves, other personal protective equipment (PPE), and hygiene practices from 69 cases and 237 controls (22 cases reported using paraquat). Unconditional logistic regression was then used to evaluate the associations between PD and pesticides, PPE, and hygiene practices and obtain stratum-specific estimates from interaction models. Based on this approach, the investigators reported no evidence of significant positive association between paraquat exposure and PD among protective glove users (OR = 1.6, 95% CI 0.6-4.2, n = 8 paraquat exposed cases reporting use of protective gloves) and evidence of a strong positive association among non-glove users, defined as report of using gloves less than 50% of the time (OR = 3.9, 95% CI 1.3-11.7, n = 14 paraquat exposed cases reporting no use of protective gloves).

Examination of Self-Reported Dream Enacting Behavior in AHS Cohort

- Shrestha et al. (2018) conducted a prospective study of the AHS cohort to examine the association between pesticide exposure, including paraquat, and dream enacting behavior. 51,350 male farmers were enrolled in the AHS between 1993-1997 and administered follow-up questionnaires in four phases to obtain follow-up information on pesticide use, potential confounders, and medical information. The most recent follow-up questionnaire was administered in 2013-2015 and included screening questions on prodromal PD symptoms including dream enacting behavior, olfactory impairment, constipation, daytime sleepiness, depression, anxiety, and several motor symptoms such as tremor and, small handwriting. 23,478 of the 51,350 (46%) male farmers originally enrolled in AHS completed this questionnaire and were the focus of Shrestha et al. (2018) analysis of the association between pesticide use and dream enacting behavior. AHS participants provided self-reported information on dream enacting behavior and were asked, "Have you ever been told, or suspected yourself, that you seem to 'act out dreams' while sleeping?" If they answered yes, they were prompted to answer additional questions on the frequency of symptoms. Pesticide use was assessed using the AHS enrollment questionnaire and focused on ever use of 49 specific pesticides, including paraquat. Enrollment data was also used on demographic and lifestyle risk factors and information on head injury was obtain from a subsequent take home questionnaire and the phase 2 follow-up questionnaire in 1999-2003. After collecting data on the outcome of interest and pesticide use, multivariable logistic regression was used to assess the relationship between pesticide exposure and dream enacting behavior, adjusting for age, smoking, alcohol consumption, marital status, education, state, and head injury. Based on this approach, the investigators reported no evidence of an association between ever-never use of paraquat and dream enacting behavior (OR = 1.1, 95% CI: 0.9-1-3, n = 339 exposed cases).

In summary, six articles examined the relationship between paraquat exposure and PD in the AHS study population. This included three primary articles that first examined the association between paraquat and either PD (Kamel et al., 2007; Tanner et al., 2011) or dream enacting behavior; Shrestha et al., 2018) and three secondary articles that were conducted as part of FAME and explored potential effect modification by dietary, behavioral factors, and genetic factors (Goldman et al., 2012; Kamel et al., 2014; Furlong et al., 2015). There was also overlap in the study population examined in each individual article regardless of whether they were identified as primary or secondary. Therefore, the results of AHS studies should not be evaluated independently. Further characterization of areas of overlap with respect to study population, follow-up, and exposure assessment methods are summarized in Table 5.2.1 below. As summarized,

Kamel et al. (2007) and the FAME nested case-control studies are based on the same study period that covered AHS enrollment during 1993-1997 through phase 2 follow-up in 1999-2003. The FAME studies clinically confirmed PD diagnosis, but the total number of paraquat exposed cases was essentially the same – 25 total exposed cases in Kamel et al. (2007) and 23 in the FAME studies – suggesting there was considerable overlap in the subjects used in this group of studies. Shrestha et al. (2018) was the only fully prospective study and included more extensive follow-up of the AHS cohort though phase 5 follow-up in 2013-2015.

Table 5.2.1. Summary of Design Elements of AHS and FAME Studies on PD and the PD Prodromal Symptom Dream Enacting Behavior.

Study	Design (# Exposed Cases)	Study Period	Exposure	Outcome
Examination of Self-Reported PD in AHS Cohort				
Kamel et al. (2007)	Cohort Cross-Sectional n = 11 incident, 14 prevalent)	1993-1997 (Enrollment) to 1999-2003 (Phase 2 Follow-up)	Ever/Never Paraquat Use	AHS Survey Instrument – ‘‘Has a doctor ever told you that you had been diagnosed with Parkinson’s disease?’
Examination of Clinically Confirmed PD in FAME Nested Case-Control Study of AHS Cohort				
Tanner et al. (2011)	Nested Case-Control n = 23 incident/prevalent	1993-1997 (Enrollment) to 1999-2003 (Phase 2 Follow-up)	Ever/Never Paraquat Use and Cumulative Lifetime Use	Agreement of two neurologists on PD diagnosis
Goldman et al. (2012)	‘’	‘’	+ Genetic Factors	‘’
Kamel et al. (2014)	‘’	‘’	+ Dietary Fat Intake	‘’
Furlong et al. (2015)	‘’	‘’	+ Use of PPE	‘’
Examination of Self-Reported Dream Enacting Behavior in AHS Cohort				
Shrestha et al. (2018)	Prospective Cohort (n = 339)	1993-1997 (Enrollment) to 2013-2015 (Phase 5 Follow-up)	Ever/Never Paraquat Use	AHS Survey Instrument – “Have you ever been told, or suspected yourself, that you seem to ‘act out dreams’ while sleeping?”

With regard to study quality, Kamel et al. (2007) was of moderate quality based on the study quality criteria outlined in the OPP framework. The primary strength of the study was that it leveraged the AHS study cohort, which provides relevant information on U.S. agricultural populations and reliable information on pesticide usage on specific pesticides rather than simply pesticide classes. The study has several limitations, however, including the lack of clinical confirmation of self-reported PD cases and a relatively small number of cases reporting use of paraquat (14 prevalent cases and 10 incident cases). The study may also be subject to recall bias if prevalent cases recall previous exposure differently than study subject without PD. This potential for bias is particularly important because Kamel et al. (2007) reported – separately – on both prevalent and incident cases, with prevalent cases reporting an OR of 1.8 (95% CI, 1.0-3.4, n = 14 paraquat exposed cases) and incident cases (for which no recall bias would be expected) reporting an OR of 1.0 (95% CI, 0.5-1.9, n = 11 paraquat exposed cases).

The FAME studies used a nested case-control design that enabled the investigators to clinically confirm PD diagnosis and obtain more detailed information on potential genetic, dietary, and occupational hygiene risk factors. For this reason, the FAME studies were determined to be of high quality based on

the OPP study quality criteria. While the FAME studies improved upon Kamel et al. (2007), particularly by confirming PD diagnosis by two neurologists, the studies appear to examine many of the same PD cases as Kamel et al. and share similar limitations, including the relatively small number of paraquat exposed PD cases (23 exposed cases) and potential for recall bias. Furthermore, the study's statistical analysis curiously combined incident and prevalent PD cases (prevalent cases would be potentially subject to recall bias and incident cases would not). This consideration is of importance because Kamel et al. (2007) previously stratified their analysis by incident and prevalent cases and reported results that suggested that recall bias (from prevalent cases) could be substantial. As such, the FAME studies do not provide additional information to help clarify this issue.

Finally, Shrestha et al. (2018) was of moderate quality and had several strengths, including its prospective design and the reliability of the AHS questionnaire to ascertain pesticide exposure for paraquat and other specific pesticides. While the study had several strengths, it was determined to be of moderate quality because of limitations in the ascertainment of the outcome dream enacting behavior and the potential risk of bias due to loss to follow-up. Ascertainment of the outcome dream enacting behavior relied on self-report by survey participants and may have introduced misclassification if participants cannot reliably report that their symptoms are consistent typical prodromal PD symptoms. Given that the study was prospective, this source of outcome misclassification is likely to be non-differential because study subjects provided information on pesticide use before reporting dream enacting behavior during Phase 5 follow-up in 2013-2015. Loss to follow-up is another important limitation because only 46% of the study subjects originally enrolled completed the Phase 4 survey in 2013-2015. This may introduce selection bias if study subject participation in the follow-up phases is related to their disease status for PD and other health outcomes.

French Agriculture and Cancer (AGRICAN) PD Study *(Low Quality: Pouchieu et al. 2018)*

Pouchieu et al. (2018) conducted a cross-sectional study within the French AGRICAN cohort, a large prospective cohort of adults involved with agriculture in France. The primary aim of AGRICAN is to assess the relationship between agricultural exposures and cancer, but the study has secondary aims that focus on other health outcomes, including respiratory and neurologic conditions. The AGRICAN study population included all adults aged 18 years and older, both active and retired, who were farm owners, farmworkers, and individuals working for companies or organizations related to agriculture (e.g., private insurance companies, banks, extension agents, foresters and gardeners, affiliated a French Health insurance system for agricultural professionals). Individuals also had to have paid at least 12 quarterly contributions to the French health insurance for agricultural professionals and be living in 2011 in one of 11 French regions with certified cancer registries.

A total of 181,842 individuals were enrolled in AGRICAN and completed a self-administered questionnaire between 2005 and 2007. The enrollment questionnaire was used to collected data on demographics, existing health conditions, lifestyle risk factors, and occupational history. For occupational history, subjects provided job history information on farm activities related to care of 5 animal types and 13 crop types. The self-reported crop history information was used to assess exposure to specific pesticide by developing a crop-exposure matrix based on French pesticide use information, including pesticide registration, sales, and recommended use practices. Self-reported PD was also ascertained in the enrollment questionnaire.

A total of 1,732 study subjects reported being diagnosed with PD by a physician (244 exposed to paraquat), representing 1.2% of the enrolled study population. Multivariable logistic regression was used to assess the association between prevalent PD and (i) self-report of working on 18 crop/livestock

categories and (ii) 14 specific pesticides, including paraquat, based on the investigators crop-exposure matrix. Based on this approach, ever/never use of paraquat was positively associated with PD in a regression model that did not adjust for potential pesticide exposure to other pesticides (OR = 1.43, 95% CI: 1.17-1.75). After adjusting for co-exposure to other pesticides; however, the investigators reported no evidence of a positive association (OR = 1.01, 95% CI: 0.41-2.49). Additional analysis was performed to assess cumulative exposure using unexposed individuals as a reference group. Based on this analysis, the investigators similarly reported no evidence of an association (1-25 years paraquat exposure – OR = 1.05, 95% CI: 0.40-2.76; 26-46 years paraquat exposure – OR = 0.94, 95% CI: 0.37-2.41).

Overall, Pouchieu et al. (2018) was of low quality based on the study quality criteria outlined in the OPP framework. While the study leveraged an existing prospective study of French agricultural workers, the study used a cross-sectional design that relied on the AGRICAN enrollment questionnaire to assess exposure and identify prevalent PD cases. As such, the study was unable to assess the temporal association between paraquat exposure and PD. The study's exposure assessment relied on the AGRICAN the study enrollment questionnaire and only asked general questions on livestock and crop categories. Pesticide exposure was then assigned using a livestock/crop-exposure matrix that relied on expert judgement. This approach was not validated and the investigators reported a high degree of correlation between pesticides (50% of correlation coefficients > 0.80), suggesting that the investigators had limited ability to evaluate paraquat and other specific pesticides in isolation. Furthermore, the study reported positive associations between each of the 18 livestock/crop categories and PD that served the basis of the pesticide exposure assessment. As such, it appears unlikely that the investigators' approach can evaluate pesticide-specific exposure to paraquat and other pesticides.

Washington State Department of Public Health PD Study *(Low Quality: Engel et al. 2001)*

The Washington State Department of Health conducted a cohort study in 1972-1976 that examined the effects of pesticide exposure on lifespan of select subpopulations within Washington State, including orchardists, pesticide applicators, pesticide formation plant workers, and other farm/agricultural workers. The "Polks Wenatchee City Directory" was used to identify unexposed subjects who were frequency matched to exposed cases by age, race, and degree of occupational physical activity. Based on this study population, Engel et al. 2001 conducted a follow-up cross-sectional study to investigate the relationship between parkinsonism and lifetime occupational pesticide exposure. Of the 1,300 original study participants, 323 were enrolled (25%), while 977 could not be enrolled because there were deceased (n=439), could not be contacted (n= 245), resided outside the study area (n=12), lost to follow-up (n=122), or refused to participate (n= 159). This included 238 exposed individuals and 72 unexposed individuals (exposure could not be determined for 13).

Each study subject received a physical examination to confirm the presence of clinical symptoms of PD. Subjects also completed a self-administered questionnaire to ascertain information on farming and pesticide use, including years of farming, crops grown, acres for each crop, pesticide use practices, application methods, and use of personal protective equipment. Generalized linear regression with a binomial distribution and log link function was then used to estimate prevalence ratios adjusting for age and smoking. Based on this approach, the investigators examined the relationship between well water use, general use of pesticides, general use of 5 pesticide classes, and use of 13 specific pesticides, including paraquat. With respect to paraquat, no evidence of an association was reported for ever/never use (Prevalence Ratio = 0.8, 95% CI 0.5 – 1.3, n = 20 exposed cases) or tertiles of years exposure (*Tertile 2 vs Tertile 1* – Prevalence Ratio = 0.4, 95% CI: 0.1- 1.4; *Tertile 3 vs Tertile 1* – Prevalence Ratio = 0.9, 95% CI: 0.4- 2.4). Similar results were also reported for tertiles of acre-years of exposure.

Overall, Engel et al. (2001) was of low quality based on the study quality criteria outlined in the OPP framework. While the study was based on a previous cohort of agricultural workers conducted in the 1970s and included physical examination to confirm the presence of clinical symptoms of PD, the participation rate was only 25% due to loss to follow-up, which may have introduced selection bias. The study also used a cross-sectional design and was unable to assess the temporal relationship between paraquat exposure and onset of PD. Finally, the study relied on a questionnaire to ascertain paraquat exposure and did not provide any information to demonstrate that it has been validated to assess cumulative exposure to paraquat or other specific pesticides.

5.2.2 Hospital-Based Study Populations

The PD systematic review literature database consisted of 17 studies on hospital-based study populations. The hospital-based study populations were recruited from rural California (8 studies), North American movement centers (1 study), the Netherlands (2 studies), Taiwan (1 study), Washington (2 studies), East Texas (1 study), and British Columbia (2 studies).

Central Valley, CA/Parkinson's Environment and Genes (PEG) Study *(Moderate Quality: Costello et al., 2009; Gatto et al., 2009; Ritz et al., 2009; Gatto et al., 2010; Wang et al., 2011; Lee et al., 2012; Sanders et al., 2017; Paul et al., 2018)*

The PEG Study used a case-control design to assess rural PD patients diagnosed in a community clinical community setting and investigate the interaction between genetics and environmental susceptibility. Study participants resided in predominantly rural communities in central California, including Fresno, Tulare, and Kern Counties and were recruited during an initial recruitment periods (2001-2007) and more recent second round of recruitment (2010-2015).

During the initial 2001-2007 study recruitment period, cases were recruited from clinics in the three counties of interest and qualified for inclusion if they were diagnosed with PD between 1998 and 2007 and lived in California for at least 5 years prior to diagnosis. Of the 563 initially eligible PD cases, 473 (84%) were examined and confirmed to have clinically 'probable' or 'possible' PD, yielding 377 PD cases. Complete demographics were not obtained for 9 cases, resulting in enrollment of 368 PD cases into the study. During the second 2010-2015 round of recruitment, the state-mandated pilot California PD registry was used to identify 4,672 PD patients living in the Fresno, Tulare, and Kern Counties. The investigators were able to contact 2,363 of these individuals and identified 581 potential cases that were eligible for the study. Of these eligible cases, 376 were enrolled in the study after examination by a movement specialist to confirm their PD diagnosis.

Control subjects were enrolled in the PEG study from 2001-2011 using two sampling strategies. The first sampling strategy was to mail letters of invitation to a selection of randomly selected residential units in each of the 3 counties. A sample of 1,212 potential controls were screened for eligibility. Eligibility criteria for controls were not having PD, being at least 35 years of age, currently residing primarily in 1 of the 3 designated counties, and living in California for at least 5 years prior to the screening. Only 1 control per household was allowed to enroll. Of the 755 eligible controls, 346 (46%) enrolled. Complete demographics were not obtained for 5 controls, resulting in enrollment of 341 controls into the study. The second sampling strategy used clustered random selection of five households that were visited in person. Based on this second approach, an additional 1,241 eligible controls were identified. Of the eligible controls, 634 declined participation and 607 controls were enrolled in the study (only 183 completed an abbreviated interview and 77 were not genotyped).

Pesticide exposure was assigned using residential history information from cases and control, combined with California pesticide use reporting data. Specifically, lifetime residential addresses were geocoded and pesticide application rates from agricultural uses (in pounds per acre per year) within 500 m of each subject's home were estimated by using a GIS-based approach that combined California pesticide use reporting data and land-use maps.

There was a total of eight articles with results on the association between paraquat exposure and PD in the PEG Study. These case-control studies are summarized below:

- Costello et al. (2009) investigated whether exposure to paraquat or maneb, alone or in combination, increases the risk of PD in a study of 368 confirmed PD cases and 341 controls aged 65 years or older. Using residential history, pesticide use reporting data, land-use maps, and GIS, as described above, residential maneb and paraquat pesticide exposures were estimated for each study participant. The assessment derived estimates of time specific (1974-1989, 1990-1999, and 1974-1999) total exposure of for maneb and paraquat. For analysis purposes maneb and paraquat exposure was categorized as no exposure, paraquat only exposure, maneb only exposure, and both paraquat and maneb exposure, for each of the time windows 1974-1999, 1974-1989, and 1990-1999. Unconditional logistic regression models were used to evaluate the association of PD with maneb and paraquat, alone or in combination. Based on this approach, the investigators reported no evidence of a positive association for paraquat only exposure (OR = 1.01, 95% CI 0.71-1.43, n = 149 exposed cases) or maneb only exposure (OR = 3.04, 95% CI 0.30 – 30.86, n = 3 exposed cases), but reported evidence of a positive association for both paraquat and maneb exposure combined (OR = 1.75, 95% CI 1.13- 2.73, n = 88 exposed cases).
- Gatto et al. (2009) investigated whether exposure to paraquat from private well water consumption in areas with historical agricultural pesticide use was associated with an increased risk of PD. Assessment of potential well water exposure was also based on CA Pesticide Use Report data, based on the same 500-m spatial buffer, and combined with self-reports of private wells as drinking water sources at a residential address. Based on this approach, the investigators used multivariable unconditional logistic regression models to analyze the data and reported no evidence of a significant positive association between paraquat exposure from well water (OR = 1.10, 95% CI 0.75-1.63, n = 79 exposed cases). This observation did not meaningfully change when exposure was stratified by low and high exposure, and after adjustment for ambient exposure (i.e., residential proximity to pesticide applications).
- Ritz et al. (2009) genotyped 324 cases of Parkinson's disease and 334 controls subjects in order to investigate gene-pesticide exposure interaction. The investigators determined polymorphisms in genes encoding the dopamine transporter (DAT) protein. The study examined paraquat/maneb combined exposure and did not specifically report results on paraquat exposure alone. [9]

[9] No substantial elevations in Parkinson's disease risk were observed among study participants with "zero/low" residential maneb and paraquat exposure, regardless of the total number of susceptibility alleles present. However, among subjects with "high" estimated residential exposure to maneb and paraquat together, estimated odds of Parkinson's disease increased with increasing number of susceptibility alleles present, relative to a reference group with no susceptibility alleles and "none/low" maneb and paraquat pesticide exposure. Odds of Parkinson's disease was not elevated among subjects with zero susceptibility alleles and "High" maneb and paraquat exposure (OR: 0.88; 95% CI: 0.22-3.48), but Parkinson's disease odds were elevated among subjects with one susceptibility allele and "high" pesticide exposure (OR: 2.99; 95% CI: 0.88-10.21), and particularly elevated among

- Gatto et al. (2010) investigated the interaction of alpha-synuclein gene (SNCA) variations with smoking and paraquat exposure. As described by the authors, several single nucleotide polymorphisms (SNPs) and haplotypes in the SNCA promoter have been observed to be associated with familial PD, so the investigators were interested in examining if there may be a gene-environment interaction that makes some individuals are more susceptible to pesticide exposure. The study used the PEG case-control design and GIS approach to estimate exposure, but only reported on paraquat even though other studies examined other pesticides. Blood and buccal samples were obtained from study subjects to determine genomic information on SNCA variants. Multivariable unconditional logistic regression was then used to calculate OR for genetic subtypes and effect modification between these subtypes. The investigators then stratified this genotype analysis by paraquat exposure using median exposure value in the control group to identify their high exposure group. Based on this approach, the investigators reported no evidence of a significant positive association between high exposure and PD, stratified by the presence of specific SNCA genotype variants (SNCA 259 Allele – OR = 1.45, 95% CI: 0.59-3.59; SNCA 263 Allele – OR = 1.35, 95% CI: 0.74-2.46, n = 31 exposed cases). The investigators also reported evidence of effect modification between the presence of the SNCA 259 allele. The investigators further explored this interaction by stratifying the analysis by age of onset (≤68 years vs > 68 years) and reported no evidence of a significant positive association in subjects with PD onset ≤68 years (OR = 3.15, 95% CI 0.74-13.37, n = 13 exposed cases), although the OR was greater than 3.0, and no evidence of an association in subjects with PD onset >68 years (OR = 0.84, 95% CI 0.27-2.62, n = 18 exposed cases).
- Wang et al. (2011) investigated the association between PD and pesticide exposure by examining workplace address as part of the general PEG GIS-based approach that used California pesticide use reporting data. Data analyses were performed using unconditional logistic regression models, adjusted for age, sex, ethnicity, education, having a 1st degree family member with PD, and smoking. As compared to those not exposed to paraquat, maneb or ziram, the investigators reported no evidence of a significant association between paraquat only and either workplace address (OR = 1.26, 95%CI 0.86-1.86, n = 81 exposed cases) or residential address (OR = 0.91, 95% CI 0.63-1.31, n = 109 exposed cases) and PD. In a combined analysis of workplace/residential address, that did not exclude exposure to maneb and ziram, the investigators reported evidence of a positive association between paraquat exposure and PD (OR = 1.50, 95%CI 1.03-2.18, n = 162 exposed cases).
- Lee et al. (2012) investigated the interaction between self-reported traumatic brain injury (TBI) and paraquat exposure in the PEG study. The paraquat exposure assessment methodology was similar to that employed by Costello et al. (2009), but also incorporated workplace address in the assessment. The data analysis included 357 cases and 754 controls and used unconditional logistic regression, adjusting for age, gender, smoking, race, county, and education, to investigate the main effects and the interaction between self-reported TBI and paraquat exposure. Based on this approach, the investigators reported evidence of a positive association between PD and paraquat exposure (OR = 1.36, 95% CI: 1.02-1.81, n = 169 exposure-cases). With respect to effect modification, the investigators reported no evidence of a significant positive interaction between paraquat exposure and TBI. Specifically, the association between TBI and PD was 1.70

those with two or more susceptibility alleles and above-the-median maneb and paraquat exposure (OR: 4.53; 95% CI: 1.70-12.09), with the latter being statistically significant.

(95% CI = 0.95-3.04) for never exposed to paraquat subjects and was 3.01 (95% CI = 1.51-6.01) for ever exposed to paraquat subjects. However, this elevation in the association between TBI vs. PD due to the paraquat exposure was not statistically significant (OR for interaction = 1.29 (95% CI = 0.52-3.19).

- Sanders et al. (2017) investigated the potential effect modification between paraquat exposure and single nucleotide polymorphisms (SNPs) in base excision repair (BER) genes. BER is a major pathway for repairing oxidative DNA damage in cells and may play a role in the susceptibility. This study was based on the original PEG case-control study, but continued enrollment through 2013 and included 619 PD cases recruited from clinics and 854 controls recruited using Medicare enrollee lists and residential tax-collector records. The study also used the same GIS-based exposure assessment approach and considered both residential and occupation address with respect to CA pesticide use land-use data. While previous PEG studies focused on other pesticides, Sanders et al.'s exposure assessment considered pesticides considered mitochondrial complex 1 inhibitors and and/or oxidative stressors as reported in Tanner at al. (2011). In order to obtain genetic information, all study subjects provided blood and saliva samples that were analyzed for SNP selection and genotype. After performing logistic regression, the investigators reported evidence of a positive association between paraquat residential/workplace exposure and PD (OR = 1.54, 95% CI: 1.23-1.93, n = 245 exposed cases). In their examination of interaction between paraquat exposure and genetic susceptibility, the investigators reported no evidence of a significant positive association between paraquat exposure in subjects with no more than 1 risk alleles (OR = 1.13, 95% CI: 0.75-1.70, n = 48 exposed cases) compared to those with 1 or fewer risk alleles and a strong positive association in subjects with 2 or more risk alleles (OR = 2.38, 95% CI: 1.44-3.95, n = 22 exposed cases). Similar associations were reported for other pesticides examined by the investigators, both in their analysis of pesticides and PD and their examination of interaction between exposure and genetic susceptibility.
- Paul et al. (2018) investigated the association between PD and symptom progression and genes that encode for Nuclear factor-erythroid 2 related factor 2 (NFE2L2 SNPs) and peroxisome proliferator activator receptor γ coactivator 1α (PPARGC1α). The study also examined exposure to paraquat/maneb combined but did not specifically report results on paraquat exposure alone.

Overall, the eight PEG studies were of moderate quality based on the study quality criteria outlined in the OPP framework. All studies relied on the same general case-control design and included similar cases and controls, although the investigators continued to enroll study subjects in subsequent studies. The primary strength of PEG was the recruitment of cases with clinically confirmed PD diagnosis. Additionally, the GIS-based approach used in the PEG studies was not subject to recall bias present in other case-control studies that relied on questionnaires to ascertain past exposure to paraquat. While PEG's recruitment of cases was a strength, controls were recruited separately using a population-based approach that relied on Medicare enrollee lists and residential tax-collector records. This approach may have introduced selection bias if cases and controls represent populations with different demographics, lifestyle factors, potential for exposure, and willingness to participate in the study. Similarly, while the GIS-based exposure approach was not subject to recall bias, reported results suggest that the approach had limited ability to investigate exposure to paraquat specifically, rather than general residential/workplace proximity to agricultural land in the three counties of interest. In addition, there is also no published information on the measurement of paraquat residue levels in residential/workplace environments or ground water. Given that this approach has not been validated, it is unclear if being present at addresses within 500 m of agricultural land can provide a reliable estimate of true exposure. The investigators also

published limited information on the correlation between different pesticides and control for co-exposure inconsistently when reporting results. The issue of correlation between pesticides is acknowledged by the investigators, but not fully examined in the analyses reported in their 8 published articles. In Gatto et al. (2009), for example, the investigators indicate that of the subjects assigned chlorpyrifos exposure based on their residential address, 91% were also exposed to paraquat. Similarly, of the paraquat exposed individuals, 73% were exposed to diazinon, 82% to methomyl, and 80% to propargite. If this degree of correlation is present in PEG, then the study may have limited ability to examine paraquat specific effects with regard to PD.

North American Multicenter PD Study *(Moderate Quality: Tanner et al., 2009)*

Tanner et al. (2009) conducted a case-control study to investigate the association between occupational and toxicant exposures and parkinsonism in eight North American movement disorder centers. Cases were recruited from the eight movement disorder centers between July 1, 2004 and May 31, 2007 and clinically evaluated using the following inclusion criteria: (1) parkinsonism of no known cause, defined as 2 or more signs (resting tremor, bradykinesia, rigidity, and postural reflex impairment), one of which must be resting tremor or bradykinesia; (2) diagnosis within 8 years to minimize the risk of survival bias; and (3) absence of dementia. Controls were frequency-matched (age, sex, location) and were either nonblood relatives or acquaintances of cases (excluding spouses) or nonblood relatives or acquaintances of other patients of the eight movement clinics. Additional controls were also recruited using a commercial list of telephone numbers.

Study subjects were informed that the aim of the study was to investigate environmental risk factors for parkinsonism. They were then interviewed using a standardized computer-assisted phone interview to collect information on potential risk factors, including questions on the use tobacco, caffeine, and alcohol, head injury, and occupational history. To determine toxicant exposure, the investigators identified specific occupations and exposures a priori and included more detailed follow-up questions in their standardized questionnaire. This included pesticide use in general and eight specific pesticides: 2,4-D, paraquat, permethrin, dieldrin, mancozeb, rotenone, maneb, and diquat. After obtaining questionnaire data on risk factors, job history, and toxicant exposures, the authors evaluated the association with occupations, job tasks, and exposures using the Wilcoxon rank sum test for continuous variables and the Fisher exact test for categorical variables. Logistic regression was also used to calculate ORs and adjust for age, sex, race/ethnicity, smoking, caffeine, alcohol, head injury, and duration of task.

A total of 519 cases and 511 controls completed the questionnaire (91% of enrollees). Based on these cases and controls, ORs are reported for 33 standard occupational categories and job tasks, including pesticide use. For pesticide use in general, the investigators reported evidence of a positive association with parkinsonism (OR = 1.90, 95% CI: 1.12-3.21, based on 44 cases). For the 44 cases reporting pesticide use, the investigators examined the association for the eight specific *a priori* pesticides and reported no evidence of a significant positive association between paraquat and parkinsonism (OR = 2.80, 95% CI: 0.81-9.72, based on 9 exposed cases). While not statistically significant, the OR estimate was moderately strong (i.e., OR > 2.0).

Overall, Tanner et al. (2009) was of moderate quality based on the study quality criteria outlined in the OPP framework. The investigators clinically confirmed PD cases, but used a more limited exposure assessment approach that relied on a questionnaire that was not validated and only enabled analysis of ever/never use of paraquat. The study may also be subject to recall bias because cases and controls may recall previous use of paraquat and other pesticides differently. Lastly, the study included only 9 PD

cases that reported paraquat use, so it may not provide a reliable effect estimate due to the small number of exposed cases.

Netherlands PD Study *(Moderate Quality: van der Mark et al., 2014; Brouwer et al., 2017)*

A hospital-based case-control study was conducted in the Netherlands to investigate risk factors associated with PD. The study initially examined possible risk reductions associated with intake of coffee, alcohol, and cigarettes, but also investigated the relationship between pesticides and PD. Cases and controls were recruited from five hospitals in four regions of the Netherlands between 2010-2012. Cases were Parkinson's disease patients identified by doctors practicing in the neurology department in each of five hospitals. For each case, two matched controls were recruited from a patient population of adults with non-neurodegenerative symptoms (median nerve neuropathy, ulnar nerve neuropathy, thoracic and lumbar disc disease, sciatica) seen at the same neurology department as each case. The investigators enrolled 444 cases and 876 controls in the study, representing 45% of eligible cases and 35% of eligible controls, respectively. Among those that provided a reason for their non-participation (50% of non-participants), a health-related excuse and non-interest were frequently cited. Cases and controls were matched on gender, age, and time-of-diagnosis, and logistic regression was used to estimate ORs and adjust for confounding.

The study was used to examine the occupational pesticide exposure (Van der Mark et al., 2014) and residential pesticide exposure (Brouwer et al., 2017). In both subsequent investigations, study authors considered pesticide use in general and specific pesticides, including paraquat. These studies are described below:

- Van der Mark et al. (2014) evaluated the association between years of occupational paraquat exposure and PD using a conditional logistic regression model that adjusted for cigarette smoking, coffee consumption, occupational skill and status, and endotoxin exposure. Exposure to paraquat was estimated by first linking participants' self-reported crops grown at their farm to a crop-exposure matrix. In this matrix, per-decade estimations are given for the percentage of farms that applied paraquat on a type of crop and the yearly frequency of application. Expert judgment regarding the probabilities and frequencies of paraquat application were provided by two former farm workers who estimated probability and frequency of use of paraquat allowed for use on potatoes, cereals, beets, maize, tulip bulbs and fruit, back to the year 1960. Estimates for other field crops, vegetables, and flowers in green houses were derived from data from Statistics Netherlands that gathered statistics on use of specific active ingredients after 1995. For periods prior to 1995, probability and frequency of application for the crops not covered by the experts were extrapolated from trends for crops for which expert estimations were available, though details were not provided. For analysis, estimated paraquat exposures were categorized into three levels: no exposure (411 cases and 818 controls), exposure between 0 and 3.8 years (18 cases and 29 controls), and exposure greater than 3.8 years (15 cases and 29 controls). Based on this approach, the investigators reported no evidence of a significant positive association. The adjusted odds ratios for association between PD and paraquat exposure were as follows, with "no exposure" being the reference group: >0-3.8 years (OR = 1.42, 95% CI: 0.71-2.85); >3.8 years (OR =1.01, 95% CI: 0.48-2.12).
- Brouwer et al. (2017) investigated the association of environmental exposure to pesticides and PD. Pesticide exposure was assessed using a spatio-temporal model that relied on residential address information and land-use data on crops in the Netherlands. Land-use datasets from each year since 1961 defined areas likely treated with specific pesticides, based on expert judgement,

within circular rings around the residential addresses, and served as a proxy for environmental pesticide exposure. For each residential address (corresponding to a subject in the study) and each pesticide, the estimated crop area present within 0-100 m (also within 0-50 m and within >50-100m) was multiplied by the estimated probability and frequency of pesticide use to estimate the total surface area in hectares (ha) treated with the pesticide during the specific year. These estimates were summed across years (up to year preceding case-diagnosis) to obtain an estimate of the subject's cumulative environmental exposures (ha-years). For control subjects, cumulative environmental exposures were calculated through the year preceding the diagnosis year of the matched case. Conditional logistic regression was used to determine adjusted ORs. Based on this approach, paraquat environmental exposure within 0-100 m of residence, there was no evidence of an association when comparing subjects ever exposed and not exposed (OR = 1.00, 95% CI 0.73 - 1.36). In further analysis based on tertiles, there was no evidence of a significant positive association among subjects in the highest exposure tertile and those not exposed (OR = 1.46, 95% CI 0.95 – 2.23) and no association in the middle exposure tertile (OR = 0.93, 95% CI 0.61 – 1.40) or low exposure tertile (OR = 0.74, 95% CI 0.47 – 1.16). A test of trend among the tertiles was not statistically significant (p=0.19).

Van der Mark et al. (2014) and Brouwer et al. (2017) assessed occupational and non-occupational paraquat exposure, respectively, and were of moderate quality based on the study quality criteria outlined in the OPP framework. Both studies utilized the same underlying dataset from a hospital-based case-control study that recruited cases and controls from the same hospital neurology departments. While this recruitment approach was a strength of the studies, participation was relatively low, with 45% of eligible cases and 35% of eligible controls participating. In addition, Van der Mark et al. (2014) assessed potential occupational paraquat exposure using a crop-exposure matrix and Brouwer et al. (2017) assessed potential environmental paraquat exposure by linking residential address to land-use data. Both these approaches relied on expert judgement to assign paraquat usage to specific crop types and may be subject to misclassification. Additionally, the GIS-based exposure approach used in Brouwer et al. (2017) lacked land-use data on pesticide application and instead estimated exposure more generically using spatial crop information and expert judgement on the frequency/probability of specific pesticide use these crops. As with the PEG studies, this approach may be limited in assessing exposure to paraquat specifically if there is a strong degree of correlation across pesticides. The investigators did not adjust for pesticide co-exposure in their statistical analysis, but reported a median Spearman correlation coefficient of 0.63 (range 0.36-1.00) for the 21 pesticides that were examined in their primary analysis. For paraquat specifically, the median Spearman correlation coefficient was 0.43 (range 0.36-0.99), based on values reported in Figure A1 of Appendix to Brouwer et al (2017), suggesting correlation across pesticides was present in their study.

Taiwan PD Study *(Moderate Quality: Liou et al., 1997)*

Liou et al. (1997) conducted a hospital-based case-control study and evaluated duration of paraquat exposure among other environmental risk factors for Parkinson's disease in Taiwan. Parkinson's disease cases (n=120) and controls (n=240) were selected from patients at the National Taiwan University Hospital in Taipei between July 1993 and June 1995. Controls were matched to cases on age and sex. Assessment of duration of past paraquat exposures (among other pesticide exposures) was based on self-report using a survey administered during a structured interview. After obtaining data, conditional logistic regression was used to estimate ORs for paraquat and other risk factors of interest. Based on this approach, the investigators reported no evidence of an association in the 1-19 years of paraquat use category (OR = 0.96, 95% CI: 0.24-3.83, n = 7 exposed cases) but evidence of a strong positive

association for the ≥20 years paraquat use category (OR = 6.44, 95% CI: 2.41-17.2, n = 24 exposed cases). The investigators more generally examined duration of herbicides/pesticides and reported no evidence of a significant positive association in the 1-19 years of use category (OR = 1.41, 95% CI: 0.52-3.85, n = 14 exposed cases) and evidence of a strong positive association for the ≥20 years use category (OR = 6.72, 95% CI: 2.62-17.21, n = 32 exposed cases). The investigators further examined the association within subjects reporting use of herbicides/pesticides and reported that participants reporting use of paraquat and other herbicides/pesticides had twice the odds of PD, compared with those who had been exposed to herbicides/ pesticides other than paraquat (OR 2.0, p-value < 0.01)

Overall, Liou et al. (1997) is of moderate quality based on the study quality criteria outlined in the OPP framework. The primary strength of the study was that it used a hospital-based case-control design to enroll PD patients as patients and match them to controls that were recruited from the same hospital. The exposure assessment, however, relied on a general questionnaire on pesticide use and may have introduced recall bias if cases and controls recall their past pesticide use differently.

Western Washington State Study *(Low Quality: Firestone et al., 2005 and 2010)*

This population-based case-control study in Western Washington State enrolled 404 incident PD cases and 526 age- and sex-matched control participants from the Group Health Cooperative (GHC) and the University of Washington. Paraquat exposure was ascertained from self-reported work histories (including job titles and industrial toxicant exposures). A panel of neurologists confirmed case status. Exposure to pesticides, including paraquat, was self-reported along with exposure to other workplace toxicants. Unconditional logistic regression models were used for both data analysis, adjusting for age, smoking status, sex (only included in the 2005 data analysis; the 2010 data analysis only included males), and ethnicity (only included in the 2010 data analysis). Firestone et al. (2005) reported no evidence of a significant positive association (OR = 1.67, 95% CI: 0.22-12.76) and Firestone et al. (2010) reported no evidence of an association (OR = 0.9, 95% CI: 0.14-5.43); however, few subjects reported paraquat use (two cases in the 2005 study and two cases in the 2010 study).

Given the small number of exposed cases (n = 2 exposed cases per study), Firestone et al. (2005 and 2010) were of low quality because they provide insufficient information on the association between paraquat exposure and PD and contributed limited weight in OPP's evaluation of findings.

East Texas Case-Control Study *(Low Quality: Dhillon et al., 2008)*

Dhillon et al. (2008) conducted a case-control study set in an East Texas population to evaluate associations between Parkinson's disease and self-reported exposure to paraquat among other pesticide products, organic pesticides, and other occupational and environmental exposures. The study base for this case-control study consisted of residents of counties located in the East Texas region. Cases (n=100) were recruited from a cohort of 800 Parkinson's disease patients followed within a neurology practice at a local medical center neurological institute located in East Texas. Inclusion criteria included the following: age 50+ years, living in the East Texas region, and completing the interview survey. Control participants (n=84) were selected from the same neurology practice as the cases, met the same inclusion and exclusion criteria, and had no history of Parkinson's disease. Participants self-reported "Ever Personally Used/Mixed or Applied" paraquat on a standardized questionnaire. The Chi-square test was used to evaluate the association between exposure and disease. The obtained odds ratio and its 95% confidence interval were not adjusted for potential confounders. Dhillon et al. (2008) reported no evidence of a significant positive association between ever having personally used, mixed, or applied paraquat and odds of Parkinson's disease (OR = 3.5, 95% CI: 0.4, 31.6). However, only 5 study participants reported

paraquat exposure (4 cases and 1 control) and the statistical power to evaluate the association was correspondingly limited.

Given the small number of exposed cases (n = 4 exposed cases) and the weakness of statistical method used for data analysis, Dhillon et al. (2008) was of low quality and provides insufficient information on the association between paraquat exposure and PD and contributed limited weight in OPP's evaluation of findings.

British Columbia Case-Control Studies *(Low Quality: Hertzman et al., 1990, 1994)*

Hertzman et al. (1990) conducted a case-control study in the rural Kootenay region of British Colombia to investigate the associations between PD and self-reported exposure to occupational and environmental exposures including paraquat. At the time of the study, Kootenay had a population of around 80,000 and forestry, agriculture, and smelting were industries in the region. The investigators identified potential cases by contacting physicians practicing in the region, and requesting that they identify their Parkinson's disease patients. These patients were then contacted and invited to participate in the study. Potential controls participants were randomly selected from electoral rolls (92% of all adult residents of the area are reportedly on the regional rolls). Potential controls were then contacted by mail and asked to complete and return the questionnaire if they were over 50 years of age. Seventy-eight percent of the potential controls (n=129) returned a completed questionnaire, and thus constitute the control group. The analysis was, however, restricted to cases and controls between age 50 and 79 years of age (57 cases and 122 controls). Hertzman et al. (1990) only had four exposed cases and no exposed controls so the study population, so the study could not calculate an effect estimate or adjust for confounding.

Given the small number of exposed cases (n = 4), Hertzman et al. (1990) was low quality and provides insufficient information on the association between paraquat exposure and PD and contributed limited weight in OPP's evaluation of findings.

Hertzman et al. (1994) conducted a second case-control study of PD in the Okanagan Valley of British Columbia, which is a horticultural region with a population of approximately 200,000 at the time of the study. The study aimed to build on the previous work reported in Hertzman et al. (1990) by focusing on a region where they expected there be a high prevalence of pesticide use in orchards. The study population consisted of PD cases who were identified by contacting physicians in the region, including 160 general practitioners, 3 neurologists, and 25 internal medicine specialists (6 doctors refused to participate). Based on this recruitment approach, 159 potential cases were identified and 142 cases were enrolled in the study after medical examination to confirm their PD diagnosis. Two control groups were included in the study. The first consisted of individuals aged 45-80 years were randomly selected from electoral rolls which were estimated to cover 92% of the regional population and be representative of the Okanagan general population. The second control group consisted of individuals with chronic cardiac disease, who were also recruited through regional physicians. Participation rates in the voter control group (n = 124 study subjects) and chronic cardiac disease patients (n = 121 study subjects) were 61% and 79%, respectively. All cases and controls were interviewed to obtain information on personal, occupational, and chemical exposure. This included 79 different pesticides that were used in the orchard industry in the region. Statistical analysis was then performed to calculate ORs of exposure to occupational exposure to different chemicals, including paraquat. The specific statistical approach was not provided, but the investigators do report that they computed Fisher exact test statistics and used a hierarchical analysis to model exposure by individual chemical, chemicals used together, and chemical classes. Based on this approach, the investigators reported no evidence of a significant positive between paraquat exposure and PD, based on either control group (PD Cases vs. Voter Control Group – OR = 1.25, 95% CI: 0.34-4.63; PD Cases vs

Chronic Cardiac Disease Controls – OR = OR = 1.11, 95% CI: 0.32-3.87, n = 6 exposed cases. However, there were only six exposed cases, so paraquat exposure appears to be very limited in the investigator's study population.

Given the small number of exposed cases (n = 6), Hertzman et al. (1994) was of low quality and provides insufficient information on the association between paraquat exposure and PD and contributed limited weight in OPP's evaluation of findings.

5.2.3 PD Registry-Based Study Populations

Nebraska PD Registry Study *(Low Quality: Wan and Lin, 2016)*

Wan and Lin (2016) conducted an ecologic study that investigated the association between county-level incidence of PD in Nebraska and country-level pesticide exposure, including paraquat, based on GIS land-use and pesticide usage data. The study utilized the Nebraska PD registry to identify PD cases and characterize their spatial distribution and county-level incidence. Nebraska established a PD registry as a result of 1996 state legislation that requires reporting of new Parkinson's cases diagnosed since January 1, 1997, although the registry also includes prevalence data on persons with PD diagnosis before 1997. The Nebraska Department of Health and Human Services maintains the PD registry and uses various sources to identify, including physician-required reports on patients who are newly diagnosed with PD within 60 days of diagnosis and semiannual reporting from pharmacies on patients who received 1 or more anti-PD medications. Based on this approach, 6,557 PD incidence cases were identified from 1997 through 2008. County-level exposure was estimated by the investigators using a GIS-based approach that combined 2005 land-use data on 19 major crop categories in Nebraska with county-level pesticide use information on 20 pesticides, including paraquat. Nebraska, however, does not maintain data on pesticide use information, so usage was derived using annual estimates from USGS. After estimating county-level PD incidence and pesticide usage, the investigators performed OLS linear regression at both the county-level and by further grouping counties based on a spatial analysis used to identify hot spots/cold spots. Based on this approach, the investigators reported no association between country-level PD incidence and any of the pesticides investigated (quantitative results not reported). The second analysis introduced a dummy variable into their regression model that adjusted for a reported hotspot of 4 counties where the incidence of PD was observed to be higher. This second analysis was conducted separately for each of 20 pesticides and stratified by quartile of exposure. Rate ratios were not calculated, but the investigators report their regression coefficients relative to quartile 1 for each pesticide. The investigators report statistically significant coefficient for Quartile 3 and 4 of paraquat exposure, but not Quartile 2 (Q2 vs Q1: 0.343, $p > 0.05$); Q3 vs Q1: 0.255, $p < 0.05$; Q4 vs Q1: 0.231, $p < 0.05$). The investigators, however, only highlighted findings that exhibited an increase an PD incidence as quartile of exposure increased, which did not include paraquat.

Overall, Wan and Lin (2016) was of low quality based on the study quality criteria outlined in the OPP framework. The primary reason for this determination is that the study used an ecologic design that does not provide individual-level information on paraquat exposure and PD. A summary of the key effect estimates from these studies is provided in Figure 5.3.3 at the end of this section.

In addition to the general limitation of the study's ecologic design, the exposure assessment approach was limited with respect to evaluating paraquat exposure because it relies on generic information on land-use data and pesticide use data. In addition, the study used OLS linear regression to evaluate the association between PD incident rate and various pesticides and other factors. It is generally more appropriate to use

Poisson regression to analyze count and rate data, so there appear to be issues with the investigators' statistical approach.

5.3 Evaluation of Findings from Human Studies

The association between paraquat exposure and PD was investigated in 13 study populations that may have been exposed to paraquat as a result of their occupation or living in rural communities that are in close proximity to agricultural land where paraquat may have been applied. OPP's evaluation of findings and overall conclusions on the association between paraquat exposure and PD are summarized in the sections below for occupational and non-occupational study populations. Occupational and non-occupational study populations are discussed separately because these populations are likely to be exposed through different exposure pathways that vary in terms of magnitude, frequency, and duration. Occupational study populations, in particular, are more likely to experience exposure as a result of direct use of paraquat in agriculture, whereas non-occupational study populations may be exposed to lower-level environmental concentrations.

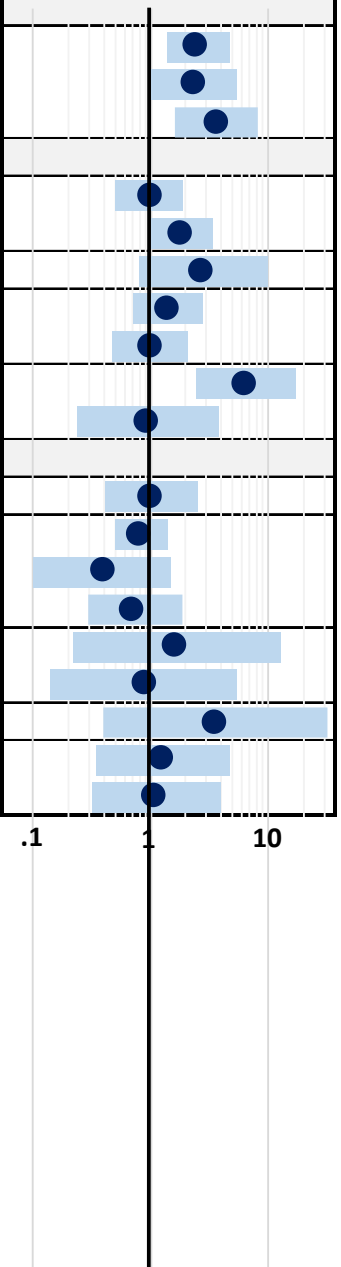
5.3.1 Occupational Paraquat Exposure

The relationship between occupational paraquat exposure and PD was investigated in 11 study populations, including AHS/FAME, the French AGRICAN, a follow-up study of the cohort by the Washington State Department of Public Health, and eight hospital-based studies. A summary of the primary findings on these study populations, including design, results, and assessment of quality, is provided in the Table 5.3.1.1 below.[10]

[10] Secondary articles further explore potential effect modification by environmental, dietary, and behavioral factors, but do not provide additional, independent information on the association between paraquat and PD. Secondary articles further expand upon, characterize, and extend the findings of primary articles and are summarized in Figure 5.3.3.1.

Table 5.3.1.1. Summary of the Primary PD Findings from Occupational Study Populations, including Design Elements, Results, and Assessment of Quality, grouped by quality rating.

Study Population	Primary Article	Design	Exposure	Outcome	Comparison	# Exposed Cases	Effect Estimate (OR, 95% CI)
High Quality							
AHS/FAME [1]	Tanner, et al., 2011	Nested Case-Control	Questionnaire	Clinical Exam	Ever/Never (Incident + Prevalent Cases)	23	
					≤Median (8 Lifetime Days)	10	
					> Median (8 Lifetime Days)	13	
Moderate Quality							
AHS	Kamel, et al., 2007	Cohort/Cross-Sectional	Questionnaire	Questionnaire	Ever/Never (Incident Cases)	11	
					Ever/Never (Prevalent Cases)	14	
NA Multicenter	Tanner et al., 2009	Case-Control	Questionnaire	Clinical Exam	Ever/Never	9	
Netherlands	van der Mark, et al., 2014	Case-Control	Questionnaire	Clinical Exam	>0-3.8 years	18	
					>3.8 years	15	
Taiwan	Liou, et al., 1997	Case-Control	Questionnaire	Clinical Exam	≥20 years using paraquat/Never	24	
					1-19 years using paraquat/Never	7	
Low Quality							
French AGRICAN	Pouchieu et al. 2018	Cross-Sectional	Questionnaire	Questionnaire	Ever/Never (Adjusted)	244	
WA Dept Public Health	Engel et al. 2001	Case-Control	Questionnaire		Ever/Never	20	
					Second Tertile (vs Tertile 1)		
					Third Tertile (vs Tertile 1)		
Western WA State	Firestone, et al. (2005)	Case-Control	Questionnaire	Clinical Exam	Ever/Never	2	
	Firestone, et al. (2010)	Case-Control	Questionnaire	Clinical Exam	Ever/Never	2	
East Texas	Dhillon, et al., 2008	Case-Control	Questionnaire	Clinical Exam	Ever/Never	4	
British Columbia	Hertzman et al., 1994	Case-Control	Questionnaire	Clinical Exam	Ever/Never (Voter Controls)	6	
					Ever/Never (Disease Controls)	6	



[1] Secondary articles included: Goldman et al., 2012; Furlong et al., 2015; Kamel et al., 2014.

The AHS (Kamel et al, 2007) and FAME (Tanner et al, 2011) studies provide the most relevant evidence on the association between paraquat and U.S. exposure and were designated to be of moderate and high quality, respectively. Both of these studies were based on the AHS study cohort and had overlap in the PD cases that were included in their analysis. Their primary strengths included AHS's focus on agricultural exposure in the U.S. and ability to recruit exposed and unexposed individuals from well-characterized agricultural populations in Iowa and North Carolina. The AHS studies also obtained information on demographic and lifestyle factors that could act as confounders and further explored potential effect modification of genetic factors and occupational hygiene practices. The results of AHS and FAME provide some evidence of a positive association between self-reported paraquat use and PD; however, the investigators reported somewhat conflicting findings for incident and prevalent PD cases. Specifically, AHS Kamel et al. (2007) study reported a non-significant positive association with *prevalent* cases, but no association with *incident* cases. This is relevant to the evaluation of evidence because the prevalent cases are more likely to be subject to recall bias if self-reported pesticide use is not independent from their previous diagnosis of PD. For example, PD cases may be subject to recall bias if they recall past exposure more accurately or incorrectly self-report the use of paraquat relative to controls. The Tanner et al (2011) FAME study, nested within AHS, does not help clarify this issue because the investigators did not examine incident and prevalent PD cases separately in their statistical analysis. Moreover, FAME may also have introduced additional recall bias because a separate exposure assessment was conducted after cases and controls were enrolled in the study.

The Tanner et al (2011) FAME study results provide additional characterization of the potential relationship between paraquat exposure and PD in AHS. First, the investigators further stratified their analysis using median duration paraquat use and observed the OR increase from 2.4 (95% CI: 1.0-5.5, n = 10 exposed cases) in individuals reporting ≤ median duration of 8 lifetime days of paraquat use to 3.6 (95% CI: 1.6-8.1, n = 13 exposed cases) in individuals reporting > median lifetime days of paraquat use. However, this analysis does not constitute a formal analysis of the dose-response relationship between paraquat exposure and PD. Moreover, the number of exposed individuals in each category was relatively small and there is no rationale provided for using the median of 8 lifetime days of paraquat use as a cut-point for making comparisons. This latter consideration is relevant because it is unclear that 8 lifetime days of exposure is biologically meaningful in terms of the magnitude and frequency of exposure. Second, the FAME investigators examined several potential effect modifiers and reported that the OR for paraquat exposure increased when also considering (i) genetic susceptibility, (ii) decreased consumption of dietary intake of fats that may be protective of PD, and (iii) use of PPE (*i.e.,* gloves) when handling pesticides. However, any causal association with these factors has not been established, some factors may also be subject to recall bias (dietary intake and PPE), and the number of exposed study subjects was small. As such, further replication of results is needed in other study populations to have confidence in these findings.

AHS conducted a prospective study and reported no evidence of an association between ever-never use of paraquat and dream enacting behavior in a more recent prospective study based on phase 4 follow-up of the AHS in cohort in 2013-2015 (Shrestha et al., 2018). This study did not evaluate PD directly and is not summarized in Table 5.2.1 above, but was determined to be moderate quality and collected information on self-reported dream enacting behavior based on follow-up of the AHS cohort in 2013-2015. The relationship between dream enacting behavior and other non-motor symptoms is an area of active research in clinical and epidemiologic research. The AHS, for example, has more generally examined the association between non-motor symptoms and PD based on cross-sectional analysis of 191 men who reported physician-diagnosed PD during phase 4 follow-up (Shrestha et al., 2017). While this analysis was cross-sectional, a strong dose-response relationship between prevalence of PD and prevalence of

dream-enacting behavior was observed amongst men in the AHS study cohort: specifically, using men reporting no dream-enacting behavior in the AHS cohort as a reference group, the ORs of reporting physician-diagnosed PD increased with frequency of dream enacting behavior: *< 3 times in life* – OR = 3.9 (1.7-8.9), n = 6 prevalent PD cases*; < once per month* – OR = 5.2 (3.1-8.5), n = 18 prevalent PD cases; *1 – 3 per month* – OR = 15.6 (9.2-26.4), n = 18 prevalent PD cases*; ≥ Once per week* – OR = 19.2 (11.0-33.5), n = 17 prevalent PD cases. This avenue of inquiry in the AHS may be useful to further continue, but suggests at this time that there is no evidence of an association between paraquat exposure and prodromal PD symptom dream enacting behavior.

The two other agricultural study populations identified included the French AGRICAN cohort (Pouchieu et al., 2018) and Washington State Department of Public Health study population (Engel et al., 2001). Both studies reported no evidence of an association; however, they were given limited weight in OPP's assessment of the epidemiologic literature studies because they had important limitations (e.g., cross-sectional design of Pouchieu et al., 2018, and 25% participation rate in Engel et al., 2001) and were both determined to be of low quality.

Eight hospital-based case-control studies examined potential occupational paraquat exposure and PD. Five of these studies were low quality and were given limited weight in OPP's assessment (Firestone et al. 2005 and 2010; Dhillon et al., 2008; and Hertzman et al., 1990 and 1994). Results of the remaining three studies, all rated moderate, were mixed and may be subject to recall bias, limitations in their exposure assessment approach, and potential selection bias. Liou et al (1997) reported the strongest positive association based on individuals reporting ≥20 years of paraquat use in Taiwan. A similar association was observed for use of herbicides/pesticides more generally in the Liou et al (1997) study, however, so it is unclear if the association is directly attributable to paraquat use, overall pesticide use considered more broadly, or another confounding factor correlated with reporting pesticide use. Tanner et al. (2009) also reported a non-significant positive association in their multicenter PD study. However, this reported association was based on only 9 exposed cases and was also similar to the reported associations for both other specific pesticides and pesticide use more generally. In contrast, Van der Mark et al. (2014) reported no association between occupational paraquat exposure and PD, based on self-reported crop activities and crop-exposure matrix. This approach is less likely to be subject to recall bias but may be subject to misclassification because exposure was determined by expert judgement and applied to all individuals for a particular job code/crop group.

Overall, there is limited, but insufficient epidemiologic evidence at this time to conclude that there is a clear associative or causal relationship between occupational paraquat exposure and PD. The conclusion that the overall evidence is limited, but insufficient is based on somewhat conflicting findings in the AHS cohort – with respect to incident and prevalent cases – and the potential for recall bias. The results of other studies outside AHS were also mixed and subject to limitations.

Studies of the AHS cohort, including Kamel et al. (2007) and the FAME studies from Tanner et al (2011), were determined to be the most relevant because of their focus on well-characterized agricultural populations in Iowa and North Carolina that are likely to experience agricultural exposure to pesticides. Kamel et al. (2007) reported a non-significant positive association with *prevalent* cases (OR = 1.8; 95% CI, 1.0-3.4, n = 14 paraquat exposed cases), but no association with *incident* cases (OR = 1.0; 95% CI, 0.5-1.9, n = 11 paraquat exposed cases). In contrast, the FAME study from Tanner et al. (2011) reported evidence of a moderately strong positive association for ever use of paraquat (OR = 2.5, 95% CI, 1.4-4.7, n = 23 exposed cases) considering prevalent and incident cases together (which makes interpretation difficult as both are subject to different limitations). Tanner et al. (2011) and the other FAME studies improved upon Kamel et al. (2007) by confirming PD diagnosis but were based on the same PD cases as

the Kamel et al (2007) AHS study and share similar limitations, including the relatively small number of paraquat exposed PD cases (23 exposed cases) and the potential for recall bias. In addition, the Tanner et al. (2011) in the FAME study combined incident and prevalent PD cases in its statistical analysis and thus does not provide additional clarification of the findings reported in Kamel et al. (2007). Finally, in a more recent prospective study based on follow-up of the AHS in cohort in 2013-2015, Shrestha et al. (2018) also reported no evidence of an association between ever-never use of paraquat and dream enacting behavior. Dream enacting behavior is a common precursor to PD and the lack of association between use of paraquat and dream enacting behavior as reported in Shrestha et al. (2018) provides additional characterization of potential PD risk within the AHS cohort.

No association was observed in the other agricultural study populations that included the French AGRICAN cohort and the Washington State Department of Public Health Study, although these studies were given less weight in this assessment because they had important limitations (i.e., cross-sectional design of Pouchieu et al., 2018, and 25% participation rate in Engel et al., 2001) and were determined to be of low quality. Finally, there were eight case-control studies that examined potential occupational paraquat exposure and PD. Five of these case-control studies were low quality and given limited weight in OPP's assessment. Results of the remaining three case-control studies, all rated moderate, were mixed with one study reporting evidence of a positive association (Liou et al., 1997), one study reporting a non-significant positive association based on only 9 exposed cases (Tanner et al., 2009), and one study reporting no evidence of an association (Van der Mark et al., 2014). These studies may also be subject to recall bias, limitations in their exposure assessment approach, and potential selection bias that introduce additional uncertainty.

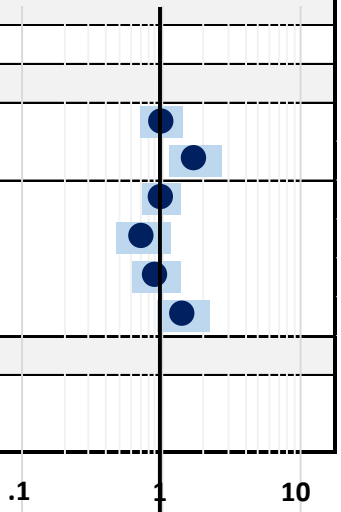
5.3.2 Non-Occupational Paraquat Exposure

The relationship between non-occupational paraquat exposure and PD was investigated in three study populations, including the PEG Study in California (8 articles), the Netherlands PD Study (1 article), and a study of the Nebraska PD registry (1 article). A summary of the primary findings on these study populations, including design, results, and assessment of quality, is provided in Table 5.3.2.1 below. [11]

[11] Secondary articles further explore potential effect modification by environmental, dietary, and behavioral factors, but do not provide additional, independent information on the association between paraquat and PD. Secondary articles further expand upon, characterize, and extend the findings of primary articles and are summarized in Figure 5.3.3.**1**

Table 5.3.2.1. Summary of the Primary PD Findings from Non-Occupational Study Populations, including Design Elements, Results, and Assessment of Quality, grouped by quality rating.

Study Population	Primary Article	Design	Exposure	Outcome	Comparison	# Exposed Cases	Effect Estimate (OR, 95% CI)
High Quality							
Moderate Quality							
CA PEG [1]	Costello, et al. (2009)	Case-Control	GIS-based Assessment	Clinical Exam	Ever/Never	149	
					Ever/Never (Paraquat+Maneb)	88	
Netherlands	Brouwer, et al. (2017)	Case-Control	GIS-based Assessment	Clinical Exam	Ever/Never	181	
					First Tertile	44	
					Second Tertile	58	
					Third Tertile	79	
Low Quality							
Nebraska PD Registry [2]	Wan and Lin, 2016	Ecologic	GIS-based Assessment	PD Registry			



[1] Secondary articles included: Gatto et al., 2009; Ritz et al., 2009; Gatto et al., 2010; Wang et al., 2011; Lee et al., 2012; Sanders et al., 2017; Paul et al., 2018.

[2] Rate ratios were not calculated, but the investigators report their regression coefficients relative to quartile 1 for each pesticide. The investigators reported statistically significant coefficient for Quartile 3 and 4 of paraquat exposure, but not Quartile 2 (Q2 vs Q1: 0.343, $p > 0.05$); Q3 vs Q1: 0.255, $p < 0.05$; Q4 vs Q1: 0.231, $p < 0.05$). The investigators, however, only highlighted findings that exhibited an increase an PD incidence as quartile of exposure increased, which did not include paraquat.

The PEG Study was of moderate quality and first examined the association between paraquat exposure and PD in Costello et al. (2009). More broadly, the PEG investigators have included analysis of paraquat in a total of eight articles that examined various measures of exposure using a GIS-based approach (residential address, residential/ workplace address) and additional questionnaire information on residential well water. Results of PEG with respect to paraquat specifically are reported in five of these eight articles and are mixed, based on different measures of exposure and consideration of co-exposure to other pesticides. Briefly:

- Costello et al. (2009) reported no evidence of an association with residential address in analysis that stratified to paraquat only exposure;
- Gatto et al. (2009) reported no evidence of an association with residential well water in analysis not stratified to paraquat only exposure;
- Wang et al. (2011) reported no evidence of an association with either residential address or workplace address in an analysis that stratified to paraquat-only exposure; however, evidence of a positive association was reported for residential/workplace address combined in an analysis that was not stratified to paraquat-only exposure;
- Lee et al. (2012) reported evidence of a positive association was reported for residential/workplace address combined in an analysis that was not stratified to paraquat-only exposure; and
- Sanders et al. (2017), which recruited additional cases through 2013, reported a positive association when they considered residential/workplace address combined in analysis not stratified to paraquat only exposure.

Additional PEG studies examined potential effect modification between pesticide exposure and other factors, including TBI (Lee et al., 2012) and genetic susceptibility (Gatto et al., 2010; Sanders et al., 2017; Paul et al., 2018). These studies make use of the same general GIS-based exposure assessment approach and may have limited ability to investigate the relationship with paraquat if there is a strong degree of correlation across different pesticides. As such, these investigations may be unable to distinguish between factors associated with geographic proximity to agricultural land and living, pesticide use in general, and specific pesticides.

The other available study on non-occupational paraquat exposure and PD was the Netherlands PD Study (Brouwer et al., 2017). This study was of moderate quality and reported no evidence of an association between paraquat exposure in their primary analysis of ever/never exposure. The investigators further stratified their analysis by tertiles of paraquat exposure and reported the highest tertile of exposure, although not significant, had the highest risk estimate. The investigators examined the trend across these tertiles and reported no evidence of a significant trend. The Netherlands PD study shares many similarities with PEG Study conducted in California. PD cases were clinically confirmed and recruited from select clinics and the exposure assessment used a GIS-based approach that was not subject to recall bias potentially present in other studies identified for this review. An additional strength of their investigation was that controls were recruited from the same neurology clinics and are more likely to represent the same underlying study population. With regard to limitations, the study had a low participation rate (45% for cases and 35% in controls and relied on a GIS-based exposure approach that lacked land-use data on pesticide application and instead estimated exposure more generically using spatial crop information and expert judgement on the frequency/probability of specific pesticide use these crops.

The remaining Nebraska PD Registry Study by Wan and Lin (2016) was of low quality because was ecologic in design and does not provide individual-level information on either paraquat exposure or the

PD outcome. As such, the study was given limited weight in OPP's evaluation of epidemiologic findings. While the study was more limited due to its ecologic design, the use of the Nebraska PD registry was a key strength that is not currently available in other U.S. states. This type of registry data is particularly helpful for characterizing the more general characteristics of PD incidence in the state of Nebraska. For example, Table 5.3.2.2, an excerpt of a table from Wan and Lin (2016), provides useful information on demographic characteristics that might be of interest when evaluating the relationship between paraquat exposure and PD. As shown, the unadjusted rate of PD incidence in Nebraska appears to be highest in counties with more poverty and greater rurality. While this rate is not adjusted for age and other factors, it suggests that rurality must be carefully considered in the design of studies that rely on GIS-based approaches.

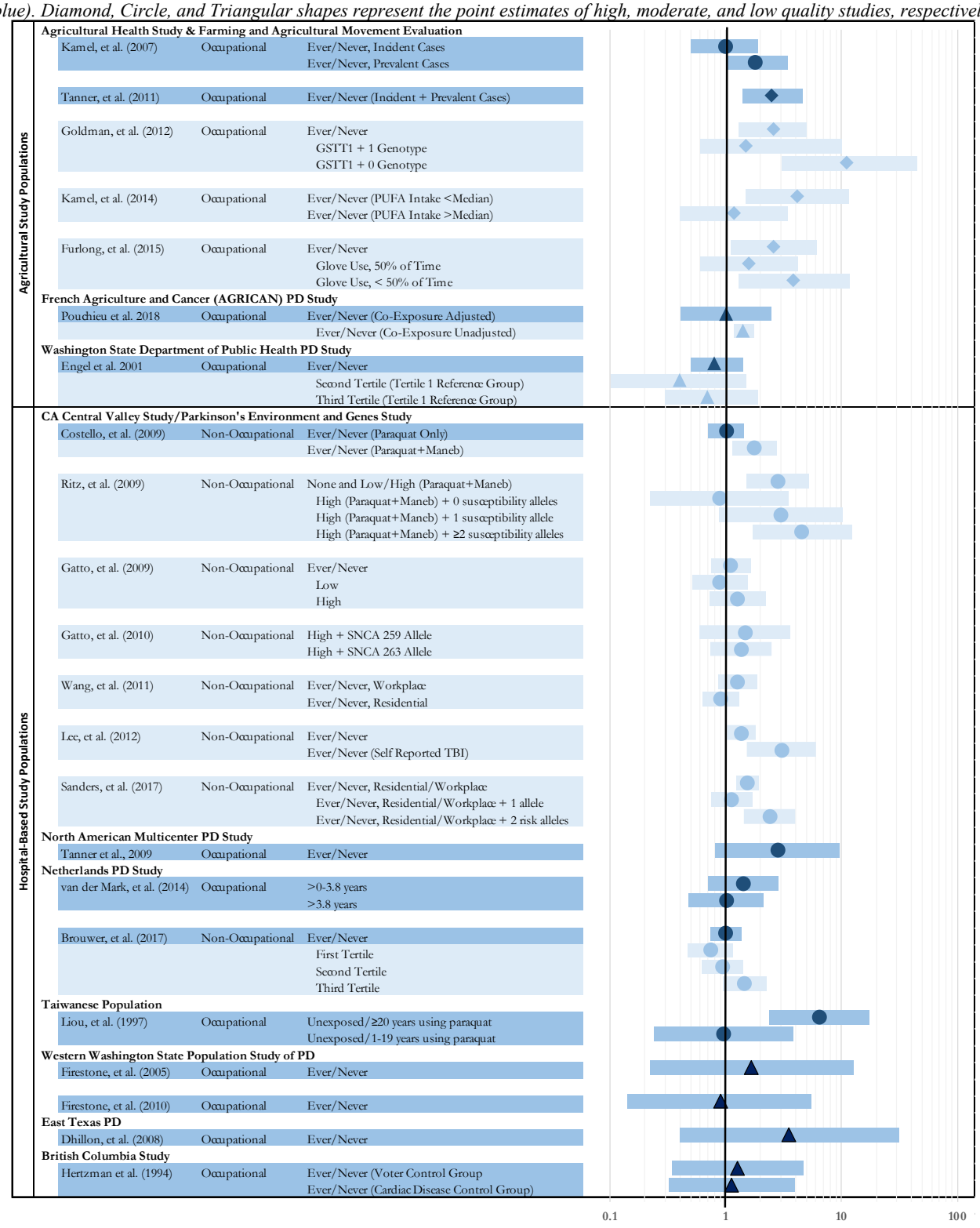
Table 5.3.2.2. Selected Characteristics of PD in Nebraska, 1997-2008 (Excerpted from Wan and Lin, 2016).

Variable	Case/Population	Rate (per Million)
Age		
Group 1 (40-64)	857/501,101	1,710
Group 2 (65-74)	1,494/115,699	12,912
Group 3 (≥75)	4,206/116,496	36,104
Poverty Rate		
Q1 (low)	3,836/1,111,956	3,450
Q2	1,414/370,492	3,817
Q3	438/87,492	4,978
Q4 (high)	869/140,831	6,171
Rurality		
Metropolitan	2,827/942,503	3,047
Micropolitan	2,561/576,660	4,441
Small town rural	66/19,450	3,393
More isolated rural	1,058/172,650	6,128

Overall, there is insufficient epidemiologic evidence at this time to conclude there a clear associative or causal relationship between non-occupational paraquat exposure and PD. This conclusion was based on the limited number of studies on non-occupational populations, lack of consistent evidence of a positive association, and the potential for bias in the available studies. The PEG study reported evidence of positive association in some publications, for example, but reported no evidence of an association when restricting analysis to paraquat exposure only. The Netherlands PD study also reported no evidence of a positive association (Brouwer et al., 2017). Moreover, both the PEG and Netherlands PD studies relied on GIS-based approaches to estimate exposure which eliminated the potential for recall bias but may have limited ability to distinguish with confidence between proximity to agricultural land, pesticide exposure in general, and specific pesticides as potential PD risk factors. The results of the Nebraska PD Registry Study (Wan and Lin, 2016) was given limited weight to OPP's evaluation because of its ecologic design, but highlights the need to carefully account for rurality in the design and analysis of studies on paraquat exposure and PD.

5.3.3 Summary of Human Findings

Figure 5.3.3.1: Summary of Odds Ratio Results for Epidemiologic Studies on Paraquat and Parkinson's Disease *(Primary study results highlighted in dark blue; Secondary study results focus on extending or further characterizing the primary study results and highlighted in light blue). Diamond, Circle, and Triangular shapes represent the point estimates of high, moderate, and low quality studies, respectively.*



6.0 Animal Data Evaluation

6.1 Study Evaluation Methods

Relevant guideline and non-guideline animal studies in the OPP toxicity database were evaluated based on OECD, OCSPP, or OPP test guidelines and OPP policies and practices to determine if they were acceptable for risk assessment. Relevant animal studies from the open literature were critically reviewed for quality based on the OPP 2012 open literature review guidance (USEPA 2012). The guidance outlines criteria for study design, data evaluation, and reporting that the reviewer considers in determining whether published literature is acceptable and relevant for use in risk assessment. The acceptability criteria are based on general expectations for studies submitted to fulfill test guidelines and are listed in the animal open literature study evaluation table (Table S1) in the supplemental document attached to this report. Deviations from the criteria were weighed based on the extent to which they affected reviewer confidence in the data reported. A study with limitations could still be classified as acceptable under the literature guidance provided the deficiencies did not critically impact confidence in all outcomes reported. Acceptable animal studies were not further distinguished with an overall confidence rating. As discussed in Section 6.2.2 and summarized in Table 6.2.2, confidence in results reported in acceptable literature studies varied based on the outcome and, in several cases, was influenced by reporting of similar outcomes in other literature studies, neither of which could not be adequately captured in a singular confidence rating for a study. Given the complexity inherent in evaluating confidence in multiple outcomes within and across studies and to confer greater transparency on the evaluation process, each deficiency and its impact on applicable outcomes was detailed and confidence in each outcome was discussed on an individual study level and in context of the weight of evidence rather than providing an overall confidence rating for each acceptable study. Studies classified as acceptable were, however, further differentiated based on whether they contribute quantitative (e.g. can be used to establish a point of departure) or qualitative (e.g. sufficient quality but cannot be used quantitatively) information to the risk assessment. Studies classified as unacceptable indicate a complete lack of confidence in all reported outcomes and, accordingly, were excluded from further consideration in the PD systematic review. Study quality evaluation also included considerations of environmental relevance – that is the likelihood that a given effect would result from an exposure scenario anticipated to occur from typical use of registered paraquat products – to determine if a study was appropriate for risk assessment.

Many of the animal studies reviewed for the PD systematic review conducted assays and assessments that are not common in the studies required for pesticide registration and thus are not covered in the test guidelines or the OPP literature review guidance. Prior to study quality evaluation, OPP solicited input from neuropathology and PD experts at NTP on questions related to the pathology of PD and the conduct of behavioral and neuropathology assessments that are often seen in PD research to aid in evaluation and interpretation of the animal studies. The discussion with the NTP experts is summarized in Appendix A.3.

6.2 Results of Study Quality Evaluation

The OPP toxicity database and NTP scoping review collectively identified 220 relevant animal studies. The four non-guideline studies submitted to the agency contained data that were also reported in published literature identified in the NTP scoping review (Breckenridge *et al.* 2013; Minnema *et al.* 2014). The non-guideline study reports included data that were not presented in the published article; therefore, the non-guideline study report and published article were considered together for study evaluation. In addition, HED was notified of a new animal study (Anselmi *et al.* 2018) published after completion of the NTP scoping review. The study was considered relevant for the systematic review topic

in accordance with the NTP scoping review screening criteria and was included in the animal evaluation. In total, 217 animal studies were evaluated for the PD systematic review.

Of the 217 studies evaluated, 21 describe toxicity in a traditional mammalian model using a route of administration that reflects an anticipated exposure scenario for pesticidal uses of paraquat (e.g. oral, dermal, or inhalation). One study (Ait-Bait et al. 2016) identified in the NTP scoping review did not report the method of administration and it could not be inferred from the methods. The remaining studies administered paraquat via injection either into the peritoneum (IP), into the subcutaneous layer (SC), or directly in the brain (IC), or investigated neurotoxicity in alternative mammalian models (e.g. *Drosophila*, nematodes, and zebrafish).

Although injection is commonly employed to elicit a PD-like response in the laboratory, it does not reflect an environmentally relevant exposure scenario for pesticidal uses of paraquat. Furthermore, toxicokinetic data indicate exposure to paraquat through injection is not analogous to ingestion. Rodents demonstrate higher peak plasma concentration (10.8-25.7 mg/L compared to 4.8 mg/L) and total absorption (80-98% compared to 6-14% based on urine data) when paraquat is administered SC and IP compared to ingestion despite similar rates of absorption (Dey *et al.* 1990; Breckenridge *et al.* 2013; Murray and Gibson 1974; Daniel and Gage 1966; Hughes *et al.* 1973). Lower systemic absorption following ingestion results in lower paraquat tissue burden in the brain and regions of the brain associated with PD compared to injection administration. Accumulation in the midbrain from acute IP exposure was approximately 2 times the midbrain tissue burden quantified after acute oral exposure to an equivalent dose (Prasad et al. 2007). An analogous comparison for repeat dosing studies was not possible due to differences in exposure regimen (e.g. IP exposure is based on number of doses rather than daily dose); however, the brain tissue concentration reported for mice exposed to 6 doses of 10 mg ion/kg over 3 weeks (0.54 ng/mg tissue; Smeyne et al. 2016) exceeded the whole brain concentration predicted for 13 weeks of dietary exposure to 10.2 mg ion/kg/day in the same strain of mouse (0.42 ng/mg tissue; Minnema et al. 2014) using a physiologically-based pharmacokinetic (PBPK) model. This is not a perfect comparison, but it does demonstrate that IP exposure to a similar dose level at lower frequency and across a shorter exposure period results in greater paraquat accumulation in brain tissues compared to oral exposure.

Data from the open literature (Chui et al. 1988; Dinis-Oliveria et al. 2008) and OPP toxicity database (Maibach 1982) also suggest absorption will be low for dermal or inhalation exposure. Chui et al. (1988) remarked that a majority of the paraquat administered via inhalation or dermal routes was present at the site of administration during the tissue distribution analysis. The authors further demonstrated that dermal bioavailability was lower than oral. Though brain tissue burden data were not available for dermal exposures, intact skin is known to be a formidable barrier to absorption (Maibach 1982) and it is anticipated that brain tissue concentrations will not exceed that observed for ingestion provided contact exposure does not compromised the integrity of the skin.

EPA acknowledges that multiple animal studies show PD-like effect when paraquat is administered via injection; however, given the divergence in toxicokinetic behavior from anticipated routes of exposure, toxicity data reported for injection studies is of limited use to assessing human risk from pesticidal uses of paraquat. Several studies identified in the NTP scoping review also explored paraquat neurotoxicity in alternative mammalian models. OPP does not currently have a policy to integrate alternative mammalian model data into the risk assessment; therefore, these studies were also considered to be of limited use for risk assessment. OPP did not conduct comprehensive quality evaluations on studies that administered paraquat via injection, did not report the method of administration, or were conducted in alternative

mammalian models due to their limited relevance to human health risk assessment. These studies were also excluded from the weight of evidence analysis.

A comprehensive quality evaluation was performed on the 21 studies identified in the OPP toxicity database (2) and the open literature (19) that investigated routes of exposure consistent with anticipated exposure scenarios for pesticidal uses of the paraquat (e.g. oral, dermal, and inhalation) and used traditional mammalian models. OPP considered these studies to be the most relevant for assessing human health risk from pesticidal uses of paraquat. It should be noted that some of the studies (e.g. Prasad et al. 2007) included information that was not relevant for risk assessment (e.g. a component of the study examined toxicity following injection exposure). These portions of the study were not evaluated for quality and were not included in the weight of evidence analysis. The results of the study quality review for studies from the OPP toxicity database and the open literature are summarized in Sections 6.2.1 and 6.2.2, respectively.

6.2.1 OPP Toxicity Database

The 2 guideline studies form the OPP toxicity database satisfied the requirements for their respective test guidelines (OCSPP 870.6200) and did not contain major deficiencies that would impact OPP's confidence in the data reported. Neither study endeavored to examine PD-like hallmarks *per se*; however, through routine behavioral assays and neuropathological assessments, the study authors reported apical outcomes that relate to PD pathology and are relevant to the weight of evidence analysis.

6.2.2 Open Literature Studies

Each of the 19 risk assessment relevant studies identified in the open literature were deficient in at least one aspect according to the 2012 OPP literature review guidance, though most deficiencies were considered minor limitations and did not impact OPP's level of confidence in the data reported. The most common deficiencies – limited product information, reporting data for a single dose, limited reporting on husbandry conditions, reporting data for a single sex, reporting qualitative information only, and potential for bias – and their impact on reviewer confidence and study classification are discussed below. The other deficiencies detailed below – uncertainties in exposure analysis, inadequate or unreported sample size, and inadequate or lack of a comparator – were observed in either a single study or across a smaller subset of studies in the database but are discussed because they had a notable impact on confidence in some or all outcome data reported in those studies.

Product Identification

Currently registered paraquat pesticidal products (technical and formulations) range in purity from 30 to 44% and chemical companies sell paraquat in several forms and at different levels of purity for scientific research. Studies that provide limited information on the paraquat product made it difficult to determine the identity of the product used due to the diversity of products available. With the exception of Naudet et al. (2017), none of the studies provided a registration or product number to identify the paraquat product. In lieu of registration or product number, purity was used to assess the likelihood that the outcomes could be attributed to paraquat exposure. The reviewer had higher confidence in studies reporting high purity (Minnema et al. 2014) or purity in the range of the currently registered paraquat products and products used in guideline studies (Widdowson et al. 1996). Endo et al. (1988) was the only study that explicitly reported using a paraquat formulation. Although the source, purity, and name of the formulation were provided, confidence in these data were lower than for studies that reported using the technical product or high purity products because the authors did not identify the components of the formulation and the purity

was below the range of currently registered paraquat products. The results of this study were attributed to the formulation as opposed to paraquat itself.

A majority of the studies failed to report purity and/or source of the paraquat product used in the study. The lack of purity information was considered only a minor deficiency if the reviewer could estimate the purity based on the identifying information provided in the methods. Nine studies (Rojo et al. 2007, Prasad et al. 2007 and 2009, Ren et al. 2009, Caroleo et al. 1996, Lou et al. 2016, Satpute et al. 2017, Luty et al. 1997, and Anselmi et al. 2018) reported sourcing the paraquat product from Sigma-Aldrich but did not provide purity information or a product number in the publication. The lack of purity in these studies did not impact confidence in the reported results, however, as the reviewer could assume the products were high purity given that Sigma-Aldrich only sells paraquat products with reported purity of ≥98%. OPP was less confident in results from studies reporting low purity or no purity information and limited or no additional identifying information.

Six studies did not report enough information to adequately characterize the paraquat product used in the study. Four studies (Chen et al. 2010; Li et al. 2015, Benzi et al. 1990; Peled-Kamar et al. 1997) failed to report both the source and purity of the paraquat product used. Fredriksson et al. (1993) reported the source (Imperial Chemical Industries), but not the purity of the product. Although the paraquat product was sourced from the same company as another study (Widdowson et al. 1996), Fredriksson et al. (1993) did not report enough information to determine if the products are identical and, if not, what product they used. The reviewer was also unable to independently verify the purity range of products manufactured and sold by Imperial Chemical Industries. Gorkin et al. (1994) did report purity information for their paraquat product but noted that it was a gift from a professor and did provide the name of the product which left the identity of it uncertain. Moreover, the purity of the product (1.25-5%) used by Gorkin *et al.* (1994) is well below the range of registered paraquat products. Although this was not the only deficiency in these studies, it is inextricably linked to the outcomes of interest reported. Without knowledge of the paraquat product, the outcomes of the study could not be unequivocally attributed to paraquat or a specific paraquat formulation and introduced considerable uncertainty into the review of these data and diminished confidence in the results. Consequently, the six animal studies that did not adequately describe the paraquat product (Chen et al. 2010; Li et al. 2015, Benzi et al. 1990; Peled-Kamar et al. 1997; Fredriksson et al. 1993; Gorkin et al. 1994) were excluded from consideration in the data evaluation and weight of evidence analysis.

Reporting Data for a Single Dose Level

Nine studies (Widdowson et al. 1996; Ren et al. 2009; Satpute et al. 2017; Naudet et al. 2017; Li et al. 2015; Benzi et al. 1990; Luty et al. 1997; Rojo et al. 2007; Anselmi et al. 2018) reported toxicity information on one or more outcomes for a single dose level only. Reporting of toxicity data for a single dose level provided an apt comparison to the concurrent control but precluded interpretation of the dose response relationship within the study. A dose response relationship could, however, be assessed in the context of analogous studies that investigated toxicity at higher and lower dose levels. Likewise, the conclusions of a single dose study would be considered more reliable when consistent with results reported at the same dose level in other acceptable studies. As a result, the impact of single dose reporting was assessed in the context of the body of evidence rather than on an individual study basis. Reporting of a single dose did not lower confidence in a given outcome provided that additional data were available from other studies to contextualize the reported results. Confidence in toxicokinetic data was not diminished when only one dose was reported.

Animal Husbandry

Reporting on husbandry conditions varied from study to study, and none of the studies provided the entire suite of information that is recommended in the OCSPP test guidelines. Nevertheless, a lack of husbandry reporting was not considered a major deficiency unless unusual findings were reported in control animals that could be related to poor husbandry. As none of the studies evaluated reported poor health in the controls, the lack of animal husbandry information did not lower confidence in reported data.

Reporting Data for One Sex Only

Only two of the 19 open literature studies (Li et al. 2015; Minnema et al. 2013) examined PD-like neurotoxicity in both sexes. A majority of the remaining studies reported data for males only (13 studies), two studies reported data for females only, and two did not specify the sex of the test animals. Data on both sexes are necessary to properly characterize risk because there is potential for both sexes to be exposed from typical paraquat use. This deficiency did not lower our confidence in the data reported for a given study; however, due to the prevalence of this deficiency in the open literature it was considered a major weakness for the overall database in the weight of evidence analysis.

Reporting Qualitative Data Only

Nearly every study evaluated reported at least one outcome qualitatively (e.g. representative images for histopathology). Confidence in the reliability of data for these outcomes was lowered if quantitative data on the same outcome were not available to verify the results reported. Reporting only qualitative information for outcomes of interest was not considered a reason for exclusion; however, confidence in these data were lower relative to other studies that reported quantitative data for the same outcome.

Bias

Evaluation of bias relied on explicit reporting and was often informed by assumptions if certain aspects of study design were not mentioned. Based on the information reported in the publication, all animal studies evaluated were determined to be potentially influenced by one or more forms of bias outlined in the OHAT Risk of Bias Tool (OHAT 2015). None of the studies reported blinding research personnel to study group during exposure or concealing allocation of animals to the different groups at the beginning of the study. According to the OHAT Risk of Bias Tool (OHAT 2015), failing to blind research personnel to study group could change how animals are handled and monitored which could ultimately impact the outcome reported. Likewise, allocation concealment is necessary to prevent selective assignment of animals to treatment and control groups and not including it as part of the study design could favor larger effect sizes (OHAT 2015). Given these forms of bias may have been present in each study evaluated, it was not used to differentiate quality between studies.

Of greater consequence was the lack of reporting in several studies on procedures to randomize allocation of animals to study group (selection bias) and to restrict awareness of research personnel to the study group during outcome assessment (performance bias). Both are associated with contributing to larger effect sizes that may exaggerate the true response (OHAT 2015). Twelve studies did not report if animal allocation was randomized (Fredriksson et al. 1993; Peled-Kamar et al. 1997; Rojo et al. 2007; Prasad et al. 2009; Caroleo et al. 1996; Satpute et al. 2017; Luty et al. 1997; Naudet et al. 2017; Prasad et al. 2007; Benzi et al. 1990; Gorkin et al. 1994; Endo et al. 1988). Given that animal allocation would influence all outcomes assessed, this potential form of bias lowered our confidence in all data reported in these studies. Likewise, nine studies did not report blinding one or more outcome assessments (Widdowson et al. 1996; Rojo et al. 2007; Ren et al. 2009; Caroleo et al. 1996; Lou et al. 2016; Luty et al. 1997; Naudet et al.

2017; Prasad et al. 2007; Prasad et al. 2009). Unlike animal allocation, however, the lack of blinding for an outcome assessment could be isolated to those outcomes and not necessarily affect the reliability of the other outcomes assessed. Furthermore, the objectivity of the procedures used in the outcome assessment was considered in determining the extent to which not blinding could affect the outcome result. For example, a lack of blinding did not affect confidence in data collected with the aid of automated equipment (e.g. locomotor activity assessments, neurochemistry), but did diminish confidence for outcomes assessed based on subjective counts or observations (e.g. stereology and histopathology). Because it relied on explicit reporting, inference of selection or performance bias in a study was not, on its own, reason for classifying a study as unacceptable. However, the perceived lack of blinding or lack of random allocation of animals was considered in evaluating the strength of the data and determining the contribution of a dataset to the weight of evidence.

Evidence for another form of bias, selective reporting (OHAT 2015), was observed in Naudet et al. 2016, Luty et al. 1997, and Anselmi et al. 2018. Outcomes for certain treatment groups mentioned in the methods of these studies were reported in the text without accompanying data that could be independently verified by the reviewer. In Naudet et al. (2016) and Luty et al. (1997), selective reporting was limited to the behavioral data, which diminished confidence in the results for that outcome only. In contrast, reporting of all *in vivo* outcomes from the lectin only and paraquat only treatment groups were incomplete in Anselmi et al. (2018). Confidence in all *in vivo* outcomes was diminished because the reviewer could not independently assess the data for these treatment groups and this study was accordingly classified unacceptable.

Uncertainties in Exposure Analysis

Limited exposure information reported in Luty et al. (1997) lowered confidence in all outcomes reported. The methods section did not explicitly identify the dermal doses used nor provide confirmatory analytical data on the dosing solutions. Furthermore, the authors did not indicate if the animals were washed after each dermal exposure or if steps were taken to prevent ingestion of the compound that may have remained on the skin. Consequently, the reviewer could not discount the possibility that ingestion of the compound may have contributed to the effects reported. Given these exposure uncertainties, the study was classified unacceptable.

Although there are no major deficiencies in the published Minnema et al. (2014) study, the study report submitted to the agency (Beck 2013) prior to publication indicated issues with homogeneity and stability of paraquat in the diet used in the dietary neurotoxicity study. The variability in these data call into question whether the animals were exposed to a consistent concentration of paraquat at the reported doses. The registrant could not determine if it was the diet preparation or analytical method but stated that paraquat's tendency to adsorb to surfaces was a likely cause of the variability in the homogeneity and stability analysis (Communication with Syngenta on 03/05/2018). Acceptable homogeneity and stability results were reported in other guideline studies that also discussed initial issues with concentration analysis; therefore, the inconsistent homogeneity and stability data described in the study report is still considered a critical limitation. It is clear from the report that the animals were exposed to paraquat; however, the variability observed in the test diet concentration diminished reviewer confidence in the accuracy of the reported exposure levels. A lack of confidence in the exposure analysis hindered establishing a relationship between the response (or lack thereof) and the dose level precluded direct quantitative comparisons with outcomes reported in other studies. As a result, the data reported for the dietary neurotoxicity study was not included in the data evaluation and weight of evidence analysis. The study report submitted to the agency did not include information on the toxicokinetics portion of this study; therefore, the reviewer relied on the information provided in the publication which did not report

issues with the diet preparation. It should be noted that homogeneity, stability, and concentration analysis data were not available for the other published studies.

Inadequate or Unreported Sample Size

Reviewer confidence in results was diminished for studies that failed to report the number of animals used or reported using an inadequate number of animals for one or more outcomes. Caroleo et al. (1996) did not report sample size for the neuropathology portion of the study. Given that neuropathology was the only relevant outcome assessed in the study, a lack of confidence in these data was reason to downgrade the study to unacceptable. Unlike Caroleo et al. (1996), Prasad et al. (2009) and Fredriksson et al. (1993) reported sample size for all outcomes; however, the reported sample size was considered inadequate in both studies. According to the methods, Prasad et al. (2009) exposed four animals, an adequate sample size for toxicokinetic experiments, but pooled the samples for the striatum paraquat measurements resulting in an effective sample size of one and no measure of variability. Fredriksson et al. (1993) report using a sample size of 8-12 offspring for the neurochemistry and behavior assays. In this study, the parental population was exposed to paraquat and offspring from three litters were used for the assays. OPP uses the litter rather than the individual animal as the basis for sample size for offspring endpoints, thus the effective sample size for these assays was three.

Lack of a Control Group

Rojo et al. 2007 and Anselmi et al. 2018 were considered deficient because they did not report control data in their motor activity analysis. In the Anselmi et al. (2018) study, baseline data were provided; however, this was not considered an adequate comparator to the treated animals. This was considered a major deficiency for that outcome assessment and it was excluded from data evaluation and weight of evidence analysis. The remaining outcomes assessed in Rojo et al. (2007) and Anselmi et al. (2018) included a comparator and thus were not impacted. Several studies also did not include a comparator in their toxicokinetic assessments (Prasad et al. 2007 and 2009; Minnema et al. 2014; Widdowson et al. 1996; Rojo et al. 2007). As control data are not required for toxicokinetic studies, confidence in these data was not affected by the lack of a comparator.

Nine of the 19 risk assessment relevant studies identified in the open literature (Widdowson et al. 1996; Ren et al. 2009; Satpute et al. 2017; Naudet et al. 2017; Minnema et al. 2014; Prasad et al. 2007; Endo et al. 1998; Rojo et al. 2007; Lou et al. 2016) were classified acceptable and were included in the data evaluation and weight of evidence analysis. All acceptable studies contained one or more deficiencies that affected confidence in specific outcomes or all outcomes but not to an extent that completely diminished confidence in the results and warranted a downgrade in overall classification. The remaining 10 studies (Chen et al. 2010; Li et al. 2015, Benzi et al. 1990; Peled-Kamar et al. 1997; Fredriksson et al. 1993; Gorkin et al. 1994; Luty et al. 1997; Caroleo et al. 1996; Prasad et al. 2009; Anselmi et al. 2018) were classified as unacceptable due to inadequate reporting on the paraquat product, insufficient sample size, insufficient reporting on number of animals tested, uncertainties in exposure analysis and/or insufficient data reporting and were excluded from data evaluation and the weight of evidence analysis. A summary of the study quality assessment results including limitations that affected confidence in one or more outcomes and study classification is presented in Table 6.2.2.1 below. The additional comments section provides more context on how these limitations informed the classification. More detail on the study quality criteria, the individual results of the study quality evaluation, and a full list of limitations for each study based on the OPP literature review guidance is captured in the animal open literature study evaluation table (Table S1) attached to this document.

Table 6.2.2.1. Summary of Animal Study Quality Evaluation Results

Study Citation	Limitations/Deficiencies[1]	Study Classification	Comments on Classification
Endo et al. 1988	– Did not report if animal allocation was randomized – Used a formulation	Acceptable/Qualitative	The limitation identified affected confidence in the data reported but not to an extent that would warrant a downgrade in overall study classification
Lou et al. 2016	– No assessment of swim performance or sensory function to evaluate the influence of these confounding factors on the results of the Morris Water Maze (MWM) test	Acceptable/Qualitative	The limitation identified affected confidence in specific outcomes but did not impact overall study classification
Minnema et al. 2014	– Unacceptable homogeneity and stability data for diet preparations in the dietary neurotoxicity portion of the study based on data from a Syngenta submission to OPP separate from the publication. – Homogeneity and stability data for the toxicokinetic study were not included in the separate Syngenta submission to OPP; therefore, the reviewer relied on the information reported in the publication to evaluate this portion of the study.	Acceptable/Qualitative	The limitations identified affected confidence in specific outcomes but did not impact overall study classification
Naudet et al. 2017	– Incomplete reporting of behavioral data for wildtype animals – Inadequate number of animals for behavioral assessment	Acceptable/Qualitative	The limitations identified affected confidence in specific outcomes but did not impact overall study classification
Prasad et al. 2007	– Did not report if animal allocation was randomized	Acceptable/Qualitative	The limitation identified affected confidence in the data reported but not to an extent that would warrant a downgrade in overall study classification
Ren et al. 2009	– Deficiency in reporting of motor activity data (could not confirm habituation) – Optical density rather than stereological methods used to assess dopaminergic neuron health – Single dose level tested	Acceptable/Qualitative	The limitations identified affected confidence in specific outcomes but did not impact overall study classification

Rojo et al. 2007	– Did not present control data for behavioral assessment – Did not report blinding status of behavior or neuropathology assessors – Did not report if animal allocation was randomized – Did not report statistical analysis methods – Neuropathology for rats was qualitative only	Acceptable/Qualitative	The limitations identified affected confidence in the data reported but did not critically diminish confidence in all outcomes that would warrant a downgrade in overall study classification
Satpute et al. 2017	– Single dose level tested – Did not report if animal allocation was randomized	Acceptable/Qualitative	The limitations identified affected confidence in specific outcomes but did not impact overall study classification
Widdowson et al. 1996	– Did not report blinding status of neuropathology or observational assessors – Neuropathology data presented qualitatively only – Single dose level tested	Acceptable/Qualitative	The limitations identified affected confidence in specific outcomes but did not impact overall study classification
Anselmi et al. 2018	– Did not report blinding status of neuropathology and behavior assessors – No control data presented for behavior assessment – Inadequate number of animals for the PQ only behavioral assessment – Incomplete reporting of animal data for the paraquat and lectin only treatment groups – Single dose level tested	Unacceptable	This study was classified unacceptable because data for the paraquat only and lectin only treatment groups were not completely reported in the publication
Benzi et al. 1990	– Did not report purity or source of the paraquat product – Did not report if animal allocation was randomized – Single dose level tested	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product
Caroleo et al. 1996	– Sample size for neuropathology assessment not reported – Did not report blinding status of neuropathology assessors	Unacceptable	This study was classified unacceptable due to the lack of reporting on the number of animals used in the only relevant outcome assessment (neuropathology)

	– Neuropathology data presented qualitatively only		
Chen et al. 2010	– Did not report purity or source of the paraquat product – Neuropathology data presented qualitatively only – No assessment of swim performance or sensory function to evaluate the influence of these confounding factors on the results of the Morris Water Maze test	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product
Fredriksson et al. 1993	– Insufficient number of animals tested – Did not report purity and cannot identify product based on source – Deficiencies in motor activity analysis (interval length and reporting)	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product and insufficient number of animals tested
Gorkin et al. 1994	– Reported both purity and source; however, the purity was lower than paraquat products used in the guideline studies and the reviewer could not verify the identity of the product used based on the reported information – Did not report if animal allocation was randomized – Single dose tested	Unacceptable	This study was classified unacceptable due to uncertainties surrounding the paraquat product
Li et al. 2015	– Did not report purity or source of the paraquat product – Neuropathology data presented qualitatively only – Single dose level tested	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product
Luty et al. 1997	– Selective reporting of behavioral data – Deficiencies in reporting of exposure methods (lack of specifics on dose levels, wash regimen, and efforts to reduce ingestion) – Did not report blinding status of neuropathology and behavior assessors	Unacceptable	This study was classified unacceptable due to the uncertainties in the exposure methodology

	– Did not report if animal allocation was randomized		
Peled-Kamar et al. 1997	– Did not report purity or source of the paraquat product – Inconsistencies in reporting of dosing and lack of clarity in methods and figures confounded interpretation of results	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product and inconsistencies in reporting.
Prasad et al. 2009	– Inadequate sample size	Unacceptable	Study classified unacceptable due to inadequate sample size for the toxicokinetic analysis

[1]Limitations that affected confidence in one or more outcomes and were considered in study classification are reported in this table. Minor limitations were not listed but are included in Supplemental Table S1.

6.3 Summary of Animal Study Results

Table 6.3.1 below summarizes methods, relevant results, and how limitations identified in the study quality assessment impacted confidence in the reported data for the 11 acceptable animal studies (two from the OPP database and nine from the open literature) included in the data evaluation and weight of evidence analysis.

Table 6.3.1. Summary of Relevant Outcome Results and Impact of Study Limitations for Acceptable Animal Studies

Study Citation	Model Organism	Study Design[1]	Results for Relevant Outcomes[2]	Impact of Study Limitations/Deficiencies on Confidence in Reported Outcomes[3]
Brammer 2006	**Species/Strain** Alpk:ApfSD Wistar derived rats **Sex** Male/Female **Age** 6 weeks	**Dose Levels** 0, 8.4, 25.1, and 84 mg ion/kg **Route** Oral (gavage) **Duration** Single dose	**Clinical Observations** Piloerection, pinched sides, respiratory distress, flaccidity, upward spiral curvature, ocular discharge, and mortality at 84 mg ion/kg **Neuropathology** No treatment-related effects **Motor Impairment** No treatment-related effects	No major limitations identified that would impact confidence in outcome results
Chivers 2006	**Species/Strain** Alpk:ApfSD Wistar derived rats **Sex** Male/Female **Age** 6 weeks	**Dose Levels** 0, 1.0/1.1, 3.4/3.9, and 10.2/11.9 mg ion/kg/day in M/F **Route** Oral (gavage) **Duration** Single dose	**Clinical Observations** No treatment-related effects **Neuropathology** No treatment-related effects **Motor Impairment** No treatment-related effects	No major limitations identified that would impact confidence in outcome results
Widdowson et al. 1996	**Species/Strain** Alpk:ApfSD Wistar derived rats **Sex** Male	**Dose Levels** Water or 5 mg ion/kg **Route** Oral (gavage) **Duration**	**Toxicokinetics** ↑ PQ concentration in brain tissue with repeat dosing **Neuropathology** No evidence of neuronal cell damage in SN	-Lower confidence in the neuropathology and behavioral assessment because the study did not report on the blinding status of these assessors

	Age Not Reported	Single dose or 1x/day for 14 days	**Motor Impairment** No change in open field, motor coordination, locomotor activity, or grip strength assessments **Neurochemistry** ↑* DA in striatum and ↑* noradrenaline in frontal cortex. No change in DOPAC or noradrenaline in striatum and no change in DA, DOPAC, or noradrenaline in hypothalamus.	
Ren et al. 2009	**Species/Strain** C57BL/6 mice **Sex** Male **Age** 8 weeks	**Dose Levels** Saline or 7.2 mg ion/kg/day (saline or 10 mg dichloride/kg/day) **Route** Oral (gavage) **Duration** 1x/day for 4 months	**Clinical Observations** Behavioral disorder and trembling while at rest in treated mice **Motor Impairment** ↓* horizontal activity in treated mice **Neurochemistry** ↓* striatal DA, DOPAC, and HVA in treated mice. Striatal 5-HT and 5-HIAA were not affected by treatment. **Neuropathology** ↓* in TH immunoreactivity in SN of treated mice **Oxidative Stress** ↑* lipid peroxidation biomarker (MDA) and ↓* antioxidant enzyme (SOD and GSH-Px) activity in SN tissue	-Lower confidence in neuropathology results because authors used optical density, rather than direct neuron count, to assess neuron degeneration
Rojo et al. 2007	**Species/Strain** C57BL/6 mice and Sprague-Dawley rats **Sex** Male **Age** 8 weeks	**Dose Levels** Saline, 10, 20, or 30 mg ion/kg/day (mice); saline or 10 mg ion/kg/day (rat) **Route** Intranasal **Duration** 1x/day for 30 days	**Clinical Observations** Curved position, agitated breathing and weight loss, cyanotic hands, feet and mouth, and lung damage at 20 mg ion/kg/day. One-third of the mice in the 30 mg/kg/day paraquat group died during the first week and another third exhibited truncal dystonia and fast rotation when suspended by their tail **Neurochemistry** No change in DA or DOPAC levels in striatum	-Lack of reporting on animal allocation randomization lowered confidence in all outcome results -Lack of reporting on blinding status of assessors lowered confidence in mouse and rat neuropathology

			Neuropathology No change in TH+ neuron count in either mice (20 mg ion/kg/day) or rats (10 mg ion/kg/day). Diffuse peroxidase reaction in the olfactory bulb of frontal cortex in rats (10 mg ion/kg/day) **Toxicokinetics** PQ only observed in olfactory bulb 10 mins after acute 20 mg ion/kg/day intranasal exposure. No evidence of PQ above the LOD in striatum and ventral midbrain.	-Lack of quantitative data (incidence and severity) lowered confidence in in rat neuropathology and clinical observations -Motor activity data excluded from evaluation due to lack of controls and selective reporting of treatment groups -Due to lack of statistical method description, statistical significance was not considered in the data evaluation
Lou et al. 2016	**Species/Strain** C57BL/6 mice **Sex** Male **Age** 3 or 8 weeks	**Dose Levels** Saline, 3.6, or 7.2 mg ion/kg/day (saline, 5, or 10 mg dichloride/kg/day) **Route** Oral (gavage) **Duration** 1x/day for 28 days	**Motor Impairment** No change in ability to locate platform during training phase of MWM test. During the spatial phase, dose dependent ↑ in latency to find the platform location from the training phase in all treatment group that was ↑* at 10 mg/kg/day. Treatment did not affect the number of times crossing platform location during spatial phase.	Study limitations did not impact confidence in outcomes reported
Satpute et al. 2017	**Species/Strain** Swiss mice **Sex** Male **Age** Not reported	**Dose Levels** Saline or 14.5 mg ion/kg/day (saline or 20 mg dichloride/kg/day) **Route** Oral (po) **Duration** 1x/day for 28 days	**Motor Impairment** ↓* time on rotarod **Oxidative Stress** ↓* in antioxidant enzyme activity (catalase and GSH-Px), ↓* GSH levels in brain, and ↑* in lipid peroxidation biomarker (MDA) in brain **Inflammation** ↑* production of cytokines and biomarker for neutrophil accumulation (myeloperoxidase activity) in brain tissue **Mitochondrial Dysfunction**	-Lack of reporting on animal allocation randomization lowered confidence in all outcome results

			↓* activity of electron transport chain complex I and IV in brain tissue and ↑* in frequency of mitochondria exhibiting outer membrane damage	
Naudet et al. 2017	**Species/Strain** C57BL/6 mice **Sex** Female **Age** 8 weeks	**Dose Levels** Tap water or 50 µg dichloride/mL (equivalent to ~7.2 mg ion/kg/day or ~10 mg dichloride/kg/day) **Route** Oral (drinking water) **Duration** Daily for 6, 7, or 8 weeks	**Neuroinflammation** ↑* in glial activity in the enteric nervous system **α-synuclein** No change in phosphorylation at Serine 129 of α-synuclein, previously reported to be a key event in Parkinson's disease pathogenesis	-Motor impairment data was excluded from evaluation because the number of animals tested was inadequate and the data were discussed in the text but not presented in numerical or graphical form in the publication
Prasad et al. 2007	**Species/Strain** C57BL/6 mice **Sex** Male **Age** 8-12 weeks	**Dose Levels** 10, 20, or 50 mg ion/kg/day **Route** Oral (gavage) **Duration** Single dose	**Toxicokinetics** Dose dependent ↑ PQ concentration in ventral midbrain tissue (0.05-0.30 ng paraquat/mg tissue) 6 hours after exposure.	Lack of reporting on animal allocation randomization lowered confidence in all outcome results
Endo et al. 1988	**Species/Strain** ICR mice **Sex** Male **Age** Not reported	**Dose Levels** 0, 3.12, 10.4, or 31.2 mg ion/kg/day **Route** Oral **Duration** 1x/day for 3 days	**Neurochemistry** Dose dependent ↓ in DA and NE midbrain levels in all treatment groups that was ↓* at 31.2 mg/kg/day and ≥10.4 mg/kg/day, respectively. No change in midbrain levels of DOPAC and HVA. No clear treatment-related changes in DA, DOPAC, HVA, or NE in cerebral cortex and pons/medulla oblongata. ↓* in 5-HT levels in midbrain in all dose groups and ↑* in 5-HIAA in midbrain and pons/medulla oblongata at 31.2 mg/kg/day. No change in acetylcholine in any brain region. ↑ choline levels in all brain regions that was ↑* in midbrain and pons/medulla oblongata in all treatment groups, and at ≥10.4 mg/kg/day in the cerebral cortex	-Lack of reporting on animal allocation randomization lowered confidence in all outcome results -All outcome results attributed to formulation

Minnema et al. 2014	**Species/Strain** C57BL/6J mice **Sex** Male/Female **Age** 10 weeks old	**Dose Levels** 0.3 or 1.5 mg ion/kg/day **Route** Oral (dietary) **Duration** 13 weeks	**Toxicokinetics** Dose and duration dependent ↑ PQ concentration in whole brain tissue that reaches an apparent plateau by the end of 13 weeks then declines after transfer to basal diet with estimated half-life of 21 days. No differences observed between sexes	-Neuropathology, neurochemistry, and neuroinflammation data excluded from evaluation due to uncertainty in the exposure analysis for the dietary neurotoxicity study -No limitations identified for the dietary toxicokinetics study

[1]Studies that reported doses levels in mg paraquat were assumed to be referring to the ion. Studies that reported dose levels as mg paraquat dichloride were converted to mg ion for consistency across the report and with the risk assessment.
[2]This column presents a simplified summary of data for outcomes relevant to the systematic review. Some of the outcome results presented were impacted by study deficiencies and limitations as discussed briefly in the last column and in more detail in Section 6.2.2; however, confidence in these results was not completely diminished and the results were still considered reliable. Outcome results in which confidence was completely diminished were excluded from the table.
[3]The impact of reporting outcome results for a single dose level was assessed in the context of the body of evidence rather than on an individual study basis. Therefore, the impact of this limitation on confidence in the reported outcomes is discussed in the animal data evaluation (Section 6.4) and weight of evidence analysis (Section 8.0) sections.
↑ = non-significant increase; ↑* = significant increase; ↓= non-significant decrease; ↓*=significant decrease; PQ = paraquat; DA = dopamine; DOPAC = 3,4-Dihydroxyphenylacetic acid, a metabolite of DA; HVA = Homovanillic acid, a metabolite of DA; NE = norepinephrine; 5-HT = serotonin; 5-HIAA = 5-hydroxyindoleacetic acid, a metabolite of 5-HT; MWM = Morris Water Maze; SN = substantia nigra; TH = tyrosine hydroxylase; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; GSH = glutathione; MDA = malondialdehyde

6.4 Evaluation of Findings in Animal Studies

Relevant animal data were separated into three categories for evaluation: toxicokinetics, PD hallmarks, and general toxicity. The toxicokinetic evaluation synthesizes information on the absorption, distribution, and elimination of paraquat from brain tissues and elaborates on the differences between routes of administration. This discussion also incorporates toxicokinetic information from the OPP toxicity database and open literature studies that were set aside in the NTP scoping review because they did not investigate neurotoxic outcomes. The PD hallmarks evaluation examines evidence for the four outcomes that constitute the main hallmarks of PD: changes in motor and non-motor behavior, dopaminergic neuron degeneration, depletion of dopamine striatal levels, and intraneuronal Lewy bodies inclusions. Neurodegenerative responses reported in other areas of the brain are also discussed as research suggests damage in multiple regions of the brain may be involved in the etiology of PD. Finally, the general toxicity evaluation covers other reported outcomes that are not specific to PD but describe toxicity in nervous tissues that may be related to the manifestation of PD.

Toxicokinetics

Toxicokinetic data for paraquat in laboratory animals describe a chemical that is poorly absorbed (6-14%) and efficiently eliminated after oral administration to sublethal doses (Daniel and Gage 1996). Likewise, intact skin presents a formidable barrier to entry (dermal absorption ~0.3% for humans; Maibach 1982) and inhaling paraquat is not anticipated to result in significant absorption (Chui et al. 1988). Despite these barriers, low amounts of paraquat do enter into systemic circulation. Serum concentrations peak at 2.1 and 4.8 mg paraquat/L (11 and 26 μM, respectively) in dogs and rats at their respective oral LD_{50} dose levels (Murray and Gibson 1974). Although it represents a small fraction of the administered dose, absorbed paraquat is sequestered in tissue and can be retained for a period of time after exposure ceases (Murray and Gibson 1974). Lung tissue is the major sink for absorbed paraquat which is not surprising given that respiratory toxicity is the most common response to paraquat exposure regardless of how it was administered (Dinis-Oliveria et al. 2008; D430827 W. Britton 2017). More relevant to the PD systematic review are the data demonstrating distribution to brain tissue (Widdowson et al. 1996; Minnema et al. 2014; Prasad 2007). Paraquat dichloride registered at quantifiable levels (0.01 ng/mg tissue) in rodent brain tissue after a single 5 mg/kg oral dose and increased by nearly an order of magnitude (0.09 ng/mg tissue) when oral exposure was repeated for 14 days (Widdowson et al. 1996). Whole brain tissue concentrations following 13 weeks of dietary exposure to 0.3-1.5 mg/kg ranged from 0.006-0.048 ng/mg tissue (Minnema et al. 2014) and were estimated to peak at 0.125-0.655 ng/mg tissue for 13-week dietary exposures to 2.4-21.5 mg/kg/day based on a simulation using a PBPK model developed by the authors. These data indicate prolonged oral exposure to paraquat promotes accumulation in brain tissue, but it reaches an apparent saturation point after 90 days of oral exposure (Minnema et al. 2014). After exposure ceases, paraquat is slowly eliminated from brain tissue with an estimated half-life of 21 days (Minnema et al. 2014).

Within the brain, paraquat was quantified in the midbrain region of mice after ingestion (Prasad *et al.* 2007). Ventral midbrain tissue burden ranged from ~0.06-0.26 ng paraquat/mg tissue after a single dose of 10-50 mg paraquat/kg (Prasad et al. 2007). No region-specific brain accumulation data following repeat oral exposure were reported in the acceptable studies to confirm ventral midbrain accumulation persisted with longer duration exposure or describe distribution of paraquat to other brain regions. Nevertheless, the observation of paraquat in the midbrain is particularly important for connecting environmentally relevant exposure to PD because it demonstrates that ingested paraquat is able to cross the blood-brain-barrier (BBB) and distribute to a region of the brain that is involved in the pathology of PD. In contrast to oral exposure, paraquat administered intranasally was entirely sequestered in the

olfactory bulb following acute exposure (Rojo et al. 2017), which is not protected by the BBB. No brain concentration data were available for repeat intranasal or inhalation exposure nor dermal exposure to quantify brain tissue concentration for those scenarios.

Distribution to the brain was also reported following IP and SC exposure in rodents (Naylor et al. 1995; Smeyne et al. 2016; Breckenridge et al. 2013). Naylor et al. (1995) described the rat BBB as an effective impediment to paraquat entry into the brain and did not detect quantifiable paraquat in regions of the brain associated with PD following acute SC exposure; however, more recent studies quantified paraquat in regions of the rodent brain important to PD in humans, namely the striatum and midbrain, following IP and SC exposure (Prasad et al. 2007 and 2009; McCormack and Di Monte 2003; Shimizu et al. 2001; Yin et al. 2011). Though the distinctive toxicokinetic behavior of injected paraquat described previously diminishes the relevance of these studies for the weight of evidence analysis, these data provide further evidence that absorbed paraquat can cross the rodent BBB and lends credence to the limited findings of intra-brain distribution in rodents described for oral exposure. Transport across the BBB may, however, be age and species-specific. Corasaniti et al. (1991) observed higher brain concentration in younger (2-weeks old) and older (>12 months old) rats compared to 3-month-old rats following acute SC exposure. The authors posited that the differences in brain concentrations may be related to age-dependent changes in the BBB permeability. Bartlett et al. (2009 and 2011) discovered that paraquat was excluded from regions of the rhesus macaque brain protected by the BBB after a single intravenous exposure. The utility of the data presented in both studies is limited to describing distribution after a single dose and the authors of the Bartlett et al. (2009) study concede that multiple doses could lead to intra-brain distribution. Regardless, based on the toxicokinetic data for oral exposures it is plausible that environmentally relevant exposure to paraquat could lead to accumulation in brain tissue that increases with duration of exposure and persists after exposure ends.

PD Hallmarks

Toxicokinetic data alone are not sufficient to ascribe an outcome to chemical exposure. The link between the toxicokinetic data and the manifestation of PD is forged based on the strength of empirical evidence of PD-like neurotoxicity in animal models. Most of the risk assessment relevant animal studies evaluated for the PD systematic review examined one or more of the PD hallmarks, though none investigated all of them in a single study nor quantified neuronal α-synuclein accumulation. Although most hallmarks were addressed at least once in the literature database, none of them were examined in more than six studies and the methods used to assess the outcomes, with the exception of neurochemistry, were rarely consistent across the studies. Limited data for each outcome, inconsistencies in the methods, and deficiencies in study design and reporting presented issues in interpreting findings across studies.

Of the risk assessment relevant studies in the literature database, Ren et al. (2009) conducted the most comprehensive investigation of PD-like hallmarks in a laboratory setting. The authors reported a significant decrease in ambulatory activity and conspicuous trembling in male C57BL/6J mice exposed to 7.2 mg ion/kg/day (10 mg dichloride/kg/day) orally for 4 months. These motor deficits were accompanied by a significant decrease in tyrosine hydroxylase (TH) optical density in the substantia nigra (SN) – a basal ganglia structure in the midbrain rich in dopaminergic neurons that experiences a substantial loss of function in humans diagnosed with PD – and a significant depression in the levels of striatal dopamine (DA) and dopamine metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), at the end of the exposure period. TH is the rate limiting enzyme for dopamine synthesis and is used to identify dopaminergic neurons for cell counting but can also be influenced by subcellular regulatory changes. Ren et al. (2009) did not confirm the decrease in TH density represented dopaminergic neuron

degeneration; however, the motor activity deficits and neurochemistry results are consistent with dopaminergic neuron degeneration.

The behavioral observations in the Ren et al. (2009) study were not an anomaly as PD-like behavioral changes were observed in the other studies that conducted behavioral assessments on male mice. Lou et al. (2016) observed a dose-dependent, but not age-dependent, increase in the latency to pass the platform in the spatial probe phase of the Morris Water Maze (MWM) test for C57BL/6J mice following 28 days of oral exposure at doses ≥3.6 mg ion/kg/day (≥5 mg dichloride/kg/day). The finding was significantly different from controls at 7.2 mg ion/kg/day (10 mg dichloride/kg/day); however, no change was observed in the time to find the platform in the training phase nor in the frequency of passing the platform after finding it in the spatial probe phase. These data were interpreted as general motor impairment effect rather than evidence of memory/learning disruption because the study authors did not rule out the influence of swimming or sensory impairment. Likewise, Satpute et al. (2017) reported significantly reduced motor coordination – represented as a decrease in latency to fall from the rotarod equipment – after 28 days of oral exposure to 14.5 mg ion/kg/day (20 mg dichloride/kg/day) in Swiss mice. Although there are some concerns with the data presented in Satpute et al. (2017) due to lack of reporting on randomizing animal allocation, the dose and duration relationship to the observed behavioral outcomes in these studies is consistent with the Ren et al. (2009) and Lou et al. (2016) results and collectively they suggest that repeat oral dosing for at least 28 days to doses ≥7.2 mg/kg/day will elicit PD-like motor impairment in male mice. No reliable data were available to determine if female mice exhibited a similar response.

Similar behavioral changes were not evident in rats suggesting the neurotoxic effects of paraquat are species-specific. Two neurotoxicity guideline studies from the OPP toxicity database reported no changes in motor activity or abnormal functional behavior in adult Alpk:Ap$_f$SD rats up to 15 days after acute gavage exposure to 8.4-84 mg ion/kg/day (Brammer 2006), and at the end of a 90-day dietary exposure to 1-11.9 mg ion/kg/day (Chivers 2006). Brammer (2006) did observe a series of clinical signs and mortality within 4-5 days after the acute exposure to 84 mg ion/kg/day; however, these findings were indicative of an agonal response to treatment and not considered evidence of neurotoxicity. The lone rat oral study from the open literature, Widdowson et al. (1996), also reported no evidence of motor impairment (open field, motor coordination, locomotor activity, or grip strength) after 14 days of gavage exposure to 5 mg ion/kg/day of a 33% w/w paraquat product.

Among the studies reporting behavioral changes in male mice, the Ren et al. (2009) study was alone in linking behavioral observations to neuropathological and neurochemical changes that were consistent with the pathology of PD. However, the Ren et al. (2009) study only investigated a single dose and exposure duration. The results of that study, therefore, do not provide enough information to evaluate dose and temporal concordance between the PD hallmarks and there are limited data available in acceptable studies from the literature database to fill in those gaps. None of the other acceptable studies examined dopaminergic neuron degeneration following repeated oral exposure. While there is internal consistency in Ren et al. (2009) among the PD hallmarks, there were no other data available to confirm that paraquat elicits dopaminergic neuron degeneration in the SN, which lowers confidence in this singular neuropathology result. The impact of paraquat on neurochemistry, in contrast, was examined in another study, Endo et al. (1988), across a dose range that overlapped with Ren et al. (2009). A dose-dependent decrease in midbrain DA and norepinephrine (NE) production appear to be an early indicator of neurochemical disruption in male mice following exposure to Gramoxon, a paraquat formulation with 24% w/w paraquat, at oral doses ≥3.12 mg/kg/day (Endo et al. 1988). The decreased DA and NE levels were significant at 31.2 mg/kg/day and doses ≥10.4 mg/kg/day, respectively. Interestingly, a

corresponding change in dopamine metabolites DOPAC and HVA was not observed even in the treatment group with significantly depressed DA levels. The authors did not elaborate on the origins of the depressed DA and NE levels in the midbrain or the neurological consequences that these results portend and the results could not be attributed solely to paraquat given that the paraquat product was a formulation, thus the relationship between these early onset midbrain neurochemical disruptions and the striatal deficits observed at four months in the Ren et al. (2009) study is not readily apparent. Moreover, the authors did not mention randomized allocation of animals to treatment groups, which lowers confidence in the results reported in Endo et al. (1988).

Most of the neuropathology observations in rats describe a lack of PD-like effects which align well with the null results reported for the behavioral assays. It should be noted that confidence in the rat neuropathological assessments is lower than for the mouse oral studies discussed above because many of the assessments were qualitative and none of the studies reported blinding the outcome assessor to treatment potentially introducing bias into the evaluation and reporting. No neuropathology lesions were observed in adult Alpk:Ap$_f$SD rats 15 days after acute exposure to 8.4-84 mg ion/kg/day (Brammer 2006), and at the end of a 90-day dietary exposure to 1-11.9 mg ion/kg/day (Chivers 2006). Widdowson et al. (1996), likewise, found no signs of qualitative neuronal cell damage in the SN of Wistar rats exposed to 5 mg ion/kg/day of a 33% w/w paraquat formulation for 14 days. Interestingly, Widdowson et al. (1996) report elevated DA levels in the striatum and noradrenaline levels in the frontal cortex with no change in DOPAC or noradrenaline levels in the striatum. The DA and metabolite data run counter to the striatal depletion that is characteristic of animals exhibiting PD-like neurotoxicity, which is consistent with the lack of other PD hallmarks in this study. Overall, the body of evidence for rats suggest a lack of PD-like neurotoxicity following oral exposure.

Information on PD-like outcomes following dermal and inhalation exposure was more limited than for oral exposure. Intranasal exposure to 20 mg ion/kg/day for 30 days failed to disrupt striatal DA and DA metabolite levels in mice (Rojo et al. 2007). The authors also reported no significant changes in the dopaminergic neuron count in the SN or TH immunoreactivity in the striatum (indicator of dopaminergic terminal health) of paraquat exposed mice or rats; however, the neuropathology data is considered less reliable due to the lack of reporting on blinding and the lack of quantitative data to confirm the reported rat neuropathology results. Distribution data following acute intranasal exposure in mice indicate that paraquat was entirely sequestered in the olfactory bulb. This supports the null findings for the tissues in the nigrostriatal pathway, but there are no data available for repeat intranasal dosing and no other studies were conducted using the intranasal or inhalation route of administration to confirm these results. Mice did exhibit clinical signs of distress at 20 and 30 mg ion/kg/day and mortality at 30 mg ion/kg/day; however, these findings were indicative of an agonal response to treatment and not considered evidence of neurotoxicity. No acceptable dermal studies were available in the literature database to evaluate PD-like outcomes following dermal exposure.

General Toxicity in Nervous Tissue

In addition to hallmarks specific to PD, several animal studies also reported on general toxicity in nervous system tissues that may contribute to the manifestation of the disease but are not inherently linked to it. Oxidative stress is a known mode of action for paraquat toxicity in other tissues, so it is not surprising to find evidence of oxidative stress in brain tissue, which toxicokinetic data suggest is in contact with absorbed paraquat. Ren et al. (2009) and Satpute et al. (2017) both observed an increase in parameters related to oxidative stress in the brain at doses that also elicited behavioral, and in the case of Ren et al. (2009), neurochemical and neuropathological changes. Ren et al. (2009) specifically reported an increase in lipid peroxidation and decrease in anti-oxidants in the SN. Dopaminergic neurons are notably

vulnerable to oxidative stress (Baltazar et al. 2014) and may explain the concordant decrease in TH optical density in the SN reported by Ren et al. (2009). Neuroinflammation is also thought to contribute to the sensitivity of dopaminergic neurons (Anthony et al. 2013; Baltazar et al. 2014); however, none of the acceptable studies in the database investigated glial or astrocyte reactivity in the regions of the brain associated with the pathology of PD. General inflammation was reported in the brain following 28 days of oral exposure to 14.5 mg ion/kg/day (Satpute et al. 2017). Naudet et al. (2017) also observed a significant increase in the astrocyte activity in the enteric nervous system of C57BL/6 mice following 6 weeks of oral exposure to 10 mg/kg/day. The Naudet et al. (2017) study suggests neurotoxicity from paraquat exposure is not limited to brain tissue; however, there were no additional acceptable studies in the literature database to confirm this result.

Mitochondrial dysfunction is another common mark of paraquat toxicity and is thought to occur via the inhibitory mechanisms enacted by paraquat on the electron transport chain complex I (Baltazar et al. 2014; Anthony et al. 2103). Complex I inhibition and mitochondrial dysfunction in neural tissues were recently proposed as key events in the PD adverse outcome pathway developed by the European Food Safety Authority (Terron et al. 2018). Only one study from the animal literature database, Satpute et al. (2017), explored mitochondrial dysfunction in brain tissue. Exposure to 14.5 mg ion/kg/day for 28 days elicited mitochondrial damage in brain tissue in the form of depressed complex I and complex IV activity and compromised integrity of the outer mitochondria membrane. The authors observed these effects concurrent with increased oxidative stress and inflammation, and decreased motor coordination; however, the authors did not confirm that the mechanistic and behavioral evidence was concordant with neuropathological and neurochemical alterations. It should also be reiterated that the lack of reporting on randomized animal allocation in this study and the absence of data from other animal studies on mitochondrial dysfunction lower confidence in the results reported for this outcome.

7.0 *In vitro* Data Evaluation

7.1 Study Evaluation Methods and Results

In vitro studies identified in the OPP toxicity database and open literature were categorized based on outcomes reported and reviewed for content. Given the size of the *in vitro* database, study quality was assessed only for studies that reported outcomes relevant to the weight of evidence analysis. The evaluation for *in vitro* studies was less rigorous than for human and animal studies because these data occupied a supportive role in the weight of evidence analysis. Acceptability criteria was based on the quality of the reporting on the test system, the test substance (e.g. purity, origin, and composition), and the test method.

A total of 244 studies were identified in the NTP scoping review that contained *in vitro* data describing paraquat toxicity in *in vitro* models commonly used for neurotoxicity assessments. The Anselmi *et al.* (2018) study identified after completion of the NTP scoping review reported *in vitro* results in addition to animal data. Given that it met the relevance criteria used in the NTP scoping review, it was also included in the *in vitro* evaluation. No relevant *in vitro* studies were found in the OPP toxicity database. Due to the density of *in vitro* database, data evaluation focused on determining the lowest concentration at which significant effects for each outcome were reported within the human and rodent models. A total of 50 *in vitro* studies were subjected to a comprehensive study quality evaluation. Collectively these studies described the most sensitive models for outcomes relevant to the PD systematic review. A summary of the study quality assessment results including limitations that affected confidence in one or more outcomes and study classification is presented in Table 7.1.1 at the end of this section. Individual results of the study quality evaluation and a full list of deficiencies for each study based on the OPP literature review guidance is captured in the *in vitro* open literature study evaluation table (Table S2) attached to this document.

Most studies evaluated for quality contained at least one of the deficiencies described in the animal open literature evaluation (Section 6.2.2). These deficiencies, both major and minor, and their impact on confidence in the results were addressed in an identical manner to that described for the animal study evaluation. The 34 studies that are summarized and evaluated in Section 7.2 were determined to be of sufficient quality to include in the data evaluation and weight of evidence analysis. The other 16 studies had at least one major reporting deficiency that critically diminished reviewer confidence in the results and conclusions. Seven of these studies (Uversky et al. 2001; Cristovao et al. 2012; Chun et al. 2001; Bonneh-Barkay et al. 2005; Cicchetti et al. 2005; Lertkaeo et al. 2017; Wang et al. 2006) did not explicitly state the number of replicates. It is clear in most of these studies that the results reflect averages of more than one replicate; however, the authors did not report the sample size or the form of replication (e.g. duplication within an experiment or replication of the experiment). OPP could not evaluate the strength of the trends and statistical results without knowledge of sample size; therefore, these studies were excluded from data evaluation. The other nine studies (Inzumi et al. 2014; Inzumi et al. 2015; Wang et al. 2009; Richardson et al. 2005; Zaidi et al. 2009; Chen et al. 2008, Gonzalez-Polo et al. 2007; Schmuck et al. 2002; de Roberto et al. 2016) did not provide enough information to adequately characterize the paraquat product tested. These were excluded from *in vitro* data evaluation for the same reasons studies inadequately reporting the paraquat product were excluded from the animal evaluation. The remaining studies were not reviewed for quality nor considered in the *in vitro* data evaluation for the following reasons: the results were not meaningfully different from those reported in the evaluated studies, the reported outcomes were not relevant to the weight of evidence analysis, and/or the results

indicated the *in vitro* model examined was not more sensitive than the relevant models discussed for a particular outcome.

Table 7.1.1. Summary of *In vitro* Study Quality Evaluation Results

Study Citation	Limitations/Deficiencies	Study Classification	Comments on Classification
Anandhan et al. 2016	None	Acceptable	No major limitations identified
Anselmi et al. 2018	None	Acceptable	No major limitations identified
Caputi et al. 2015	None	Acceptable	No major limitations identified
Case et al. 2016	None	Acceptable	No major limitations identified
Chang et al. 2013	None	Acceptable	No major limitations identified
Chau et al. 2010	None	Acceptable	No major limitations identified
Chinta et al. 2008	None	Acceptable	No major limitations identified
Choi et al. 2008	None	Acceptable	No major limitations identified
Choi et al. 2010	None	Acceptable	No major limitations identified
Cristovao et al. 2009	None	Acceptable	No major limitations identified
Ding and Keller 2001	None	Acceptable	No major limitations identified
Dou et al. 2016	None	Acceptable	No major limitations identified
Feng et al. 2011	None	Acceptable	No major limitations identified
Hirata et al. 1986	None	Acceptable	No major limitations identified
Huang et al. 2012	None	Acceptable	No major limitations identified
Huang et al. 2016	None	Acceptable	No major limitations identified
Kim et al. 2004	None	Acceptable	No major limitations identified
Klintworth et al. 2007	None	Acceptable	No major limitations identified
Loper et al. 2012	None	Acceptable	No major limitations identified
Manning-Bog et al. 2002	None	Acceptable	No major limitations identified
McCarthy et al. 2004	None	Acceptable	No major limitations identified
Navarro-Yepes et al. 2016	None	Acceptable	No major limitations identified
Ortiz-Ortiz et al. 2009	None	Acceptable	No major limitations identified
Ortiz-Ortiz et al. 2011	None	Acceptable	No major limitations identified
Peng et al. 2004	None	Acceptable	No major limitations identified
Peng et al. 2009	None	Acceptable	No major limitations identified
Rathinam et al. 2012	None	Acceptable	No major limitations identified
Shimizu et al. 2003	None	Acceptable	No major limitations identified
Vornov et al. 1998	None	Acceptable	No major limitations identified
Wu et al. 2005	None	Acceptable	No major limitations identified
Yang and Tiffany-Castiglioni 2005	None	Acceptable	No major limitations identified
Yang and Tiffany-Castiglioni 2007	None	Acceptable	No major limitations identified

Yang and Tiffany-Castiglioni 2008	None	Acceptable	No major limitations identified
Zhao et al. 2017	None	Acceptable	No major limitations identified
Bonneh-Barkay et al. 2005	-Did not report sample size	Unacceptable	Study classified unacceptable due to lack of reporting on sample size
Chen et al. 2008	-Did not report source or purity of paraquat product	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product
Chun et al. 2001	-Did not report sample size	Unacceptable	Study classified unacceptable due to lack of reporting on sample size
Cicchetti et al. 2005	-Did not report sample size	Unacceptable	Study classified unacceptable due to lack of reporting on sample size
Cristovao et al. 2012	-Did not report sample size	Unacceptable	Study classified unacceptable due to lack of reporting on sample size
de Roberto et al. 2016	-Did not report source or purity of paraquat product	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product
Gonzalez-Polo et al. 2007	-Did not report source or purity of paraquat product	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product
Izumi et al. 2014	-Reported source (Nacali Tesque) but not purity of paraquat product. Reviewer could not assume high purity based on products available in the Nacali Tesque catalog	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product
Izumi et al. 2015	-Reported source (Nacali Tesque) but not purity of paraquat product. Reviewer could not assume high purity based on products available in the Nacali Tesque catalog	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product
Lerkaeo et al. 2017	-Did not report sample size	Unacceptable	Study classified unacceptable due to lack of reporting on sample size
Richardson et al. 2005	-Did not report source or purity of paraquat product	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product
Schmuck et al. 2002	-Did not report source or purity of paraquat product	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product
Uversky et al. 2001	-Did not report sample size	Unacceptable	Study classified unacceptable due to lack of reporting on sample size
Wang et al. 2006	-Did not report sample size	Unacceptable	Study classified unacceptable due to lack of reporting on sample size

Wang et al. 2009	-Did not report source or purity of paraquat product	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product
Zaidi et al. 2009	-Did not report source or purity of paraquat product	Unacceptable	This study was classified unacceptable based on the insufficient reporting of the paraquat product

7.2 Summary of In vitro Study Results

Table 7.2.1 below summarizes the *in vitro* model, study design and relevant outcome results for the 34 acceptable *in vitro* studies included in the data evaluation and weight of evidence analysis. No major limitations were identified for these studies that would impact confidence in the data reported.

Table 7.2.1. Summary of Study Design and Relevant Outcome Results for Acceptable *In vitro* Studies

Study Citation	*In vitro* Model	Study Design	Results for Relevant Outcomes
Anandhan et al. 2016	**Model** N27 cells **Species** Rat	**Dose Levels** 0, 25, 50, 100, or 200 µM **Duration** 12, 24, or 48 hours	**Cell Viability** ↓* in cell survival after 48-hour exposure to ≥50 µM that was exacerbated in cells overexpressing wildtype or mutant α-synuclein **Mitochondrial Dysfunction** ↓* respiration after exposure for 12 hours to 25 µM
Anselmi et al. 2018	**Model** Recombinant α-synuclein **Species** N/A	**Dose Levels** 0 or 100 µM **Duration** ~40 hours	**α-synuclein** ↑* in rate of α-synuclein fibril formation at 100 µM [#]Rate was further accelerated when co-exposed to paraquat and lectins
Caputi et al. 2015	**Model** SH-SY5Y cells **Species** Human	**Dose Levels** 0, 50, 100, 250, 500, or 1000 µM **Duration** 24 or 48 hours	**α-synuclein** ↑* α-synuclein formation after 48-hour exposure to 100 µM. **TH Expression** ↑* after 48-hour exposure to 100 µM. **Cell Viability** Concentration and duration dependent ↓* at ≥50 µM.
Case et al. 2016	**Model** NG108-15 cells	**Dose Levels** 0, 10, 50, 100, or 500 µM	**Cell Viability** Concentration and duration dependent ↓* at ≥10 µM.

	Species Rat/Mouse hybrid	**Duration** 24, 48, or 72 hours	**Oxidative Stress** ↑* total cellular superoxide, ↑* cytoplasmic H_2O_2, slight ↑ in GSH, and ↑* in GSSG levels after exposure for 48 hours to 50 µM. **Mitochondrial Dysfunction** ↑* mitochondrial superoxide after exposure for 48 hours to 50 µM. No effect on mitochondrial H_2O_2 levels.
Chang et al. 2013	**Model** ReNcell CX cells **Species** Human	**Dose Levels** 0, 0.1, 1, 10, or 100 µM **Duration** 24 hours	**Cell Viability** ↓* in cell viability and ↑* in caspase-3 activity at 100 µM. Concentration dependent ↓* in cell proliferation and ↑* intracellular calcium release at ≥1 µM. **Oxidative Stress** Concentration dependent ↑* at ≥10 µM.
Chau et al. 2010	**Model** SH-SY5Y cells **Species** Human	**Dose Levels** 0 or 300 µM **Duration** 48 hours	**Cell Viability** ↑ caspase-3 activity and ↑* cell death at 300 µM **Oxidative Stress** ↑ reactive oxygen species production at 300 µM **Mitochondrial Dysfunction** ↓ mitochondrial membrane potential at 300 µM [#]↑ sensitivity for all parameters in cells that over-expressed A53T mutant α-synuclein compared to vector.
Chinta et al. 2008	**Model** N27 cells **Species** Rat	**Dose Levels** 0, 100, 250, or 500 µM **Duration** 12, 24, or 48 hours	**Cell Viability** Concentration and duration dependent ↓ in cell viability after 48-hour exposure to ≥250 µM. Decrease in cell viability coincided with evidence of endoplasmic reticulum stress (protein expression, p23 cleavage, increase in caspase activity with focus on 3 and 7). **Ubiquitin-proteasome System** ↓* 20S proteasome activity and ↑* ubiquitinated proteins after 48-hour exposure to 500 µM.

Choi et al. 2008	**Model** Primary mesencephalic neuron cultures **Species** Mouse	**Dose Levels** 0, 25, or 50 µM **Duration** 24 hours	**Dopaminergic Neuron Health** Concentration dependent ↓* TH+ neuron survival at ≥25 µM. **Cell Viability** Concentration dependent ↓ total neuron survival, ↓ GABA+ neurons, and ↑ total neuron apoptosis at ≥25 µM.
Choi et al. 2010	**Model** Primary mesencephalic neuron cultures **Species** Mouse	**Dose Levels** 0, 25, 40 or 50 µM **Duration** 8, 12 or 24 hours	**Dopaminergic Neuron Health** Concentration dependent ↑ TH+ neuron death after 24-hour exposure to ≥25 µM. ↑ caspase-3 protein levels after 12-hour exposure to 40 µM.
Cristovao et al. 2009	**Model** N27 cells **Species** Rat	**Dose Levels** 0, 100, 500, 800, or 1000 µM **Duration** 24 hours	**Cell Viability** Concentration dependent ↓* in cell viability and ↑* cell death at ≥500 µM. **Oxidative Stress** Concentration dependent ↑* in reactive oxygen species at ≥500 µM.
Ding and Keller 2001	**Model** SH-SY5Y cells **Species** Human	**Dose Levels** 0, 20, or 200 µM **Duration** 1, 3, 6, 12, or 24 hours	**Cell Viability** Concentration and duration dependent ↑* cell death at ≥20 µM. **Oxidative Stress** ↑* ROS levels after 6-hour exposure to 200 µM. **Ubiquitin-proteasome System** Concentration and duration dependent ↑* chymotrypsin-like proteasome activity at ≥20 µM. **Mitochondrial Dysfunction** Concentration and duration dependent ↓* mitochondrial membrane potential at ≥20 µM.
Dou et al. 2016	**Model** Human neural progenitor cells (hNPC)	**Dose Levels** 0, 1, 10, or 100 µM **Duration**	**Cell Viability** Concentration dependent ↑* cell death marker (LDH) at ≥10 µM. **Oxidative Stress**

	Species Human	24 hours	Concentration dependent ↑* lipid peroxidation biomarker (MDA) at ≥10 µM. ↓* in antioxidant enzyme (SOD and catalase) activity at 100 µM.
Feng et al. 2011	**Model** Mn9D cells **Species** Mouse	**Dose Levels** 0 or 50 µM **Duration** 24 hours	**Cell Viability** ↓* in cells over-expressing human α-synuclein exposed to 50 µM paraquat and 100 µM dopamine or paraquat 50 µM and 100 µM L-DOPA. **Oxidative Stress** ↑* HO-1 protein levels in normal cells and cells over-expressing human α-synuclein exposed to 50 µM paraquat and 100 µM dopamine. **Cell Membrane Permeability** ↑* in normal cells and cells over-expressing human α-synuclein exposed to 50 µM paraquat and 100 µM dopamine. #none of these parameters were affected by exposure to paraquat alone
Hirata et al. 1986	**Model** Striatal tissue slices **Species** Rat	**Dose Levels** 0, 0.1, 1, 10, 100, or 1000 µM **Duration** Not reported	**TH Activity** No effect
Huang et al. 2012	**Model** PC12 cells **Species** Rat	**Dose Levels** 0, 10, 30, 100, 300, 500, 1000, 1500, 3000, or 5000 µM **Duration** 24 hours	**Cell Viability** ↓* cell viability at ≥300 µM. **Mitochondrial Dysfunction** Duration dependent ↑* mitochondrial ROS, ↓* mitochondrial potential, ↑ formation of mitochondrial membrane permeability, and ↑* mitochondrial H_2O_2 at 1000 µM. **Oxidative Stress** No effect on cytosolic H_2O_2 levels at 1000 µM.
Huang et al. 2016	**Model** hNPC	**Dose Levels** 0, 25, 50, or 100 µM	**Cell Viability** Concentration dependent ↓ at ≥25 µM that was ↓* at ≥50 µM.

	Species Human	**Duration** 24 hours	**Oxidative Stress** Concentration dependent ↑ in reactive oxygen species levels at ≥25 µM that was ↑* at ≥50 µM.
Kim et al. 2004	**Model** Primary cortical neuron culture **Species** Rat	**Dose Levels** 0, 10, 30, or 50 µM **Duration** 24 hours	**Cell Viability** Concentration and duration dependent ↓* at ≥10 µM. Treated cells exhibited characteristics of apoptosis including shrunken bodies, condensed nuclei, and deteriorating dendrites.
Klintworth et al. 2007	**Model** SH-SY5Y cells, PC12 cells, or primary ventral mesencephalic cultures **Species** Human and Rat	**Dose Levels** 0, 2, 10, 20, 40, 50, 100, 150, 200, 250, or 500 µM **Duration** Up to 36 hours	**Cell Viability** Concentration dependent ↑ in SH-SY5Y apoptosis at ≥250 µM and ↓* in cell viability at 500 µM after 24-hour exposure. Concentration dependent ↑* in undifferentiated and differentiated PC12 cell apoptosis at ≥20 µM and ≥50 µM, respectively, and concentration dependent ↓* in cell viability at ≥50 µM in both undifferentiated and differentiated after 24-hour exposure. General neuron population survival of primary neuron cultures was unaffected up to 100 µM after 24-hour exposure. **Dopaminergic Neuron Health** Concentration dependent ↓* TH+ neurons in primary neuron cultures at ≥2 µM after 24-hour exposure.
Loper et al. 2012	**Model** N27 cells or E15 primary mesencephalic culture **Species** Rat	**Dose Levels** 0, 100, 300, or 1000 µM **Duration** 24 hours	**Cell Viability** Concentration dependent ↑* in primary mesencephalic culture neuron death at ≥100 µM. No change in cell viability in N27 cell line up to 300 µM. **Oxidative Stress** ↑* H_2O_2 production in primary mesencephalic cultures at 300 µM. No change in H_2O_2 production in N27 cell line up to 300 µM.
Manning-Bog et al. 2002	**Model** Recombinant α-synuclein **Species** N/A	**Dose Levels** 0, 10, 100, 500, or 1000 µM **Duration** Up to 40 hours	**α-synuclein** Concentration dependent ↑ in α-synuclein fibril formation at ≥10 µM

McCarthy et al. 2004	**Model** Differentiated SH-SY5Y cells **Species** Human	**Dose Levels** 0 or 10 µM **Duration** 48 hours	**Cell Viability** ~60% cell death and ↑ caspase-3 activity at 10 µM. Dead cells exhibited characteristics of apoptosis (e.g. nuclear condensation). **Oxidative Stress** ↑ total cell ROS, ↑ lipid peroxidation markers, and ↓ glutathione levels at 10 µM. **Ubiquitin-proteasome System** ↑ proteasome activity after at 10 µM **Mitochondrial Dysfunction** ↑ mitochondrial ROS and ↓ in ATP production at 10 µM.
Navarro-Yepes et al. 2016	**Model** SK-N-SH cells or Lund human mesencephalic neuronal precursor cells (LUHMES) **Species** Human	**Dose Levels** 0, 100, 200, 500, or 1000 µM **Duration** 48 hours	**Cell Viability** ↑ SK-N-SH cell death at ≥200 µM that was ↑* at ≥500 µM. **Ubiquitin-proteasome System** Concentration dependent ↓ ubiquitinated protein in SK-N-SH cells at ≥500 µM and in LUHMES cells at 200 µM. Concentration dependent ↑* in 20S proteasome activity at ≥100 µM, and ↑ accumulation of oxidized proteins at ≥200 in SK-N-SH cells. **Autophagy** Concentration and duration dependent ↓ autophagy flux at ≥200 in SK-N-SH cells. **α-synuclein** No effect of paraquat on endogenous levels in normal SK-N-SH cells. ↑ accumulation of unfolded and low molecular weight α-synuclein, but not high molecular weight α-synuclein in SK-N-SH cells overexpressing wildtype or mutant α-synuclein at ≥200 µM.
Ortiz-Ortiz et al. 2009	**Model** SH-SY5Y cells **Species**	**Dose Levels** 0, 5, 10, 25, 50, or 100 µM **Duration**	**Cell Viability** Concentration dependent ↓ in cell viability and ↑* cell death at ≥25 µM.

	Human	24 hours	**Oxidative Stress** ↑* superoxide generation at 100 µM.
Ortiz-Ortiz et al. 2011	**Model** Undifferentiated and differentiated MESC2.10 cells **Species** Human	**Dose Levels** 0, 10, 25, 50, 75, 100, 250, or 500 µM **Duration** 24 or 48 hours	**Cell Viability** Concentration and duration dependent ↓* in undifferentiated and differentiated cell viability after exposure ≥10 µM and ≥250 µM, respectively.
Peng et al. 2004	**Model** N27 cells or mesencephalic neuron cultures **Species** Rat or Mouse	**Dose Levels** 0, 40, 100, 200, 300, or 400 µM **Duration** 18 or 24 hours	**Cell Viability** Concentration dependent ↓ after 24-hour exposure to ≥100 µM. ↑ caspase-3 activity after 18-hour exposure to 400 µM. **Dopaminergic Neuron Health** ↓ TH+ neurons after 24-hour exposure to 40 µM.↑ caspase-3 activity after 18-hour exposure to 40 µM
Peng et al. 2009	**Model** E14-15 primary mesencephalic neuron glia, neuron enriched, or microglial cultures **Species** Mouse	**Dose Levels** 0, 0.5, 1, or 2 µM **Duration** 10-30 minutes or 5 days	**Dopaminergic Neuron Health** ↓* TH+ neurons in neuron-glia cultures at 1 µM for 5 days. No change in neuron-enriched cultures at 1 µM. **Oxidative Stress** Concentration dependent ↑* in extracellular superoxide production in microglial cultures after 20-minute exposure to ≥0.5 µM.
Rathinam et al. 2012	**Model** Primary cortical neuron or cortical astrocyte cultures **Species** Rat	**Dose Levels** 0, 1, 2, 10, 30, 50, 100, 500, 1000, or 2000 µM **Duration** 4 or 24 hours	**Cell Viability** ↓* in neuron viability after 4-hour exposure to ≥30 µM and after 24-hour exposure to ≥2 µM. ↓* in astrocyte viability after 24-hour exposure to ≥100 µM. No effect on astrocyte viability after 4-hour exposure. **Oxidative Stress** ↓* in GSH, ↑* ROS in cortical neurons after 24-hour exposure to 30 µM. ↓* in GSH, ↑* ROS in cortical astrocytes after 24-hour exposure to 500 µM.

			[#]Co-culture of primary neurons and astrocytes resulted in 1.5-5-fold increase in GSH and no evidence of reduced viability up to 2000 µM.
Shimizu et al. 2003	**Model** Organotypic midbrain culture **Species** Rat	**Dose Levels** 0, 1, 10, 25, 50, or 100 µM **Duration** 48 hours	**Dopaminergic Neuron Health** Concentration dependent ↓* in TH+ neurons at ≥50 µM
Vornov et al. 1998	**Model** Organotypic hippocampal culture **Species** Rat	**Dose Levels** 0, 10, 30, 100, 300, or 1000 µM **Duration** 12, 24, 48, 72, or 144 hours	**Cell Viability** Concentration and duration dependent ↑cell injury at ≥10 µM. **Oxidative Stress** Duration dependent ↓ in GSH from exposure to 10 µM.
Wu et al. 2005	**Model** Ventral mesencephalic neuron glia, microglia-enriched, and microglia-depleted cultures **Species** Rat or Mouse	**Dose Levels** 0, 0.5, or 1 µM **Duration** 30 minutes or 3, 6, 12, 24, 96, 144 or 168 hours	**Neurotransmitter Uptake** Concentration dependent ↓* in rat neuron-glia cultures after 144-168-hour (6-7 day) exposure to ≥0.5 µM. No effect on GABA uptake or dopamine uptake in microglia-depleted neuron cultures. **Dopaminergic Neuron Health** Concentration dependent ↓* TH+ neurons in rat neuron-glia cultures after 144-168-hour (6-7 day) exposure to ≥0.5 µM. **Oxidative Stress** ↑* superoxide production in rat microglia-enriched cultures after 30-minute exposure to ≥0.5 µM.
Yang and Tiffany-Castiglioni 2005	**Model** SH-SY5Y cells **Species** Human	**Dose Levels** 0, 50, 100, 250, 500, or 1000 µM **Duration** 6, 12, 24, or 48 hours	**Cell Viability** Concentration dependent ↓ at ≥100 µM that was ↓* at ≥500 µM after a 48-hour exposure. **Oxidative Stress** Duration dependent ↑* in peroxide levels at 500 µM. Duration dependent ↓* in GSH levels and GSH-Px activity and ↑* in GST activity at 500 µM. No effect on glutathione reductase activity. Duration dependent ↑* lipid peroxidation biomarker (MDA) and

			↑* in protein oxidative damage marker (protein carbonyls) at 500 µM. Duration dependent ↑ in HO-1 levels at 500 µM. **Mitochondrial Dysfunction** Duration dependent ↓* in mitochondrial transmembrane potential at 500 µM. **TH Protein Levels** No effect at 500 µM
Yang and Tiffany-Castiglioni 2007	**Model** SH-SY5Y cells **Species** Human	**Dose Levels** 0, 50, 100, 250, 500, or 1000 µM **Duration** 6, 12, 24, or 48 hours	**Cell Viability** Concentration dependent ↓ in cell viability and ↑ in cell death after 48-hour exposure to ≥250 µM that was ↑* ≥500 µM. **Ubiquitin-proteasome System** Duration dependent ↓* in proteasome activity, 19S proteasome subunit protein levels, and ↑* ubiquitinated proteins at 500 µM. No effect on the 20S proteasome subunit protein levels. **Mitochondrial Dysfunction** Duration dependent ↓* complex V activity and ATP levels at 500 µM. **α-synuclein** Duration dependent ↑* α-synuclein levels at 500 µM.
Yang and Tiffany-Castiglioni 2008	**Model** SH-SY5Y cells **Species** Human	**Dose Levels** 0, 50, 100, 250, 500, or 1000 µM **Duration** 6, 12, 24 or 48 hours	**Cell Viability** Concentration dependent ↑ in cytotoxicity after 48-hour exposure to ≥250 µM that was ↑* at ≥500 µM. Duration dependent ↑ caspase-3 and caspase-9 activity, ↑ visible nuclear condensation, and ↑* DNA fragmentation at 500 µM. **Mitochondrial Dysfunction** Duration dependent ↓* in mitochondrial transmembrane potential, ↓* complex I activity, ↑* cytosolic cytochrome c, and↓* mitochondrial cytochrome c at 500 µM. **Ubiquitin-proteasome System** Concentration dependent ↓ in intracellular proteasome activity after 48-hour exposure to ≥250 µM that was ↓* at ≥500 µM.

Zhao et al. 2017	**Model** SH-SY5Y cells or primary cortical neuron cultures **Species** Human or Rat	**Dose Levels** 0, 1, 2, 5, 10, 25, 50, 62.5, 100, 125, 250, 500, 1000, or 2000 µM **Duration** Up to 24 hours	**Cell Viability** ↑* SH-SY5Y cell death (measured by LDH release) after 24-hour exposure to ≥1000 µM. **Mitochondrial Dysfunction** Concentration and duration dependent ↑* SH-SY5Y cells and primary cortical neurons with fragmented, small round mitochondria at ≥125 µM and ≥2 µM, respectively. Duration dependent ↓* SH-SY5Y and primary cortical neuron mitochondria aspect ratio at 500 µM and 5 µM, respectively. Concentration dependent ↑* in mitochondrial ROS and ↓* mitochondrial membrane potential in primary cortical neurons after 24-hour exposure to ≥2 µM.

↑ = non-significant increase; ↑* = significant increase; ↓= non-significant decrease; ↓*=significant decrease; DA = dopamine; SN = substantia nigra; TH = tyrosine hydroxylase; ROS = reactive oxygen species; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; GSH = glutathione; GSSG = glutathione disulfide; GST = glutathione-S-transferase; MDA = malondialdehyde; LDH = lactate dehydrogenase;

7.3 Evaluation of Findings from *In vitro* Studies

The *in vitro* literature assessed a diverse list of outcomes across an array of human and rodent *in vitro* models. Relevant rodent *in vitro* models assessed in the literature include primary neuron cultures, organotypic slice cultures, and the PC12 and N27 cell lines. Rodent primary neuron cultures are prepared from brain tissue of rodent embryos (generally on gestation day 13 or 14). Primary neuron cultures collected from midbrain tissue contain 5-10% dopaminergic neurons and are cultured alongside cell types that reside in proximity such as glial cells (Falkenburger and Schultz 2006). Organotypic slice cultures are collected postnatally and preserve the neurons and surrounding cell types as well as neuronal projections (Falkenburger and Schultz 2006). PC12 and N27 are both immortalized cell lines. The PC12 cell line is derived from rat neuroblastoma cells whereas the N27 cell line is derived from rat midbrain neurons. Both cell lines display dopaminergic characteristics including production of DA and expression of TH and the dopamine transporter (Peng et al. 2004; Falkenburger and Schultz 2006). In addition, PC12 cells can be differentiated to more closely resemble a neuron phenotype. Relevant human *in vitro* models assessed in the literature include human neural progenitor, MESC.10, SH-SY5Y, and SK-N-SH cell lines. Human neural progenitor cell lines (hNPCs) are immortalized stem cells that are precursors to multiple neuronal cell types in the central nervous system and, under the correct conditions, can differentiate into dopaminergic neurons. MESC2.10 is an immortalized cell line cultured from 8-week-old human embryonic ventral mesencephalic tissue and SH-SY5Y cells as well as its parent cell SK-N-SH are immortalized cell lines derived from human neuroblastoma cells. SH-SY5Y and MESC2.10 cells express many of the same dopaminergic characteristics as the PC12 and N27 rat cell lines, and, similar to the PC12 cell line, develop additional neuron characteristics after differentiation (Falkenburger and Schultz 2006). Other relevant cell lines were also examined but were less sensitive than the models described above.

Most of the information reported covered outcomes that were not specific to PD including oxidative stress and mitochondrial effects. Outcomes with a direct connection to PD hallmarks such as dopaminergic neuron health, alpha-synuclein formation, and neurochemical changes were also investigated *in vitro*, but received less attention compared to the non-specific outcomes. Both types of outcomes are relevant to the weight of evidence analysis and are discussed separately below.

PD-Specific Outcomes

The impact of paraquat on alpha-synuclein (α-synuclein) was assessed using recombinant α-synuclein created in the lab as well as human and rodent cell cultures. Outside of the cell, paraquat was observed to accelerate the rate of recombinant α-synuclein fibril formation in a concentration dependent manner at concentrations $\geq$10 μM (Manning-Bog et al. 2001; Anselmi et al. 2018). In SH-SY5Y cells, α-synuclein protein levels increased after 48 hours of exposure at concentrations as low as 100 μM (Caputi et al. 2015). Several studies also examined the impact of α-synuclein overexpression on cell viability. Human (SH-SY5Y) and rat (N27) cell lines exhibited greater sensitivity to paraquat at concentrations of 300 μM and 50 μM, respectively, when the cells over expressed wildtype or A53T mutant alpha-synuclein (Chau et al. 2010; Anandhan et al. 2016). In contrast, an engineered mouse cell line, MN9Dsyn, that overexpressed wildtype α-synuclein did not exhibit increased sensitivity to paraquat at 50 μM; however, exposure to multiple oxidative stressors, in this case paraquat and dopamine, elicited a dramatic increase in cytotoxicity compared to controls (Feng et al. 2011).

Dopaminergic neuron viability was assessed directly in midbrain neuron cultures and indirectly based on the response in cell lines with dopaminergic properties. Rodent primary mesencephalic cultures obtained from rat and mouse fetal tissue were the most studied *in vitro* model for this outcome. Exposure as low as

1 μM for 5 days (Peng et al. 2009) or 0.5 μM for 6-7 days (Wu et al. 2005) resulted in a significant decrease the viability of dopaminergic neurons (measured based on TH+ immunoreactivity) in cultures collected from C57BL/6 mice and Fischer rats, respectively. Dopaminergic neuron degeneration in this model was also observed over shorter exposure durations at higher concentrations. For example, selective degeneration of dopaminergic neurons in primary ventral mesencephalic cultures prepared from Sprague-Dawley rats (Klintworth et al. 2007) was observed at doses as low as 2 μM after 24 hours of exposure. Although, Peng et al. (2009) and Wu et al. (2005) did not report blinding the assessors during the neuron count, the similar response observed by Klintworth et al. (2007) using a blinded assessment increase confidence in the results reported by Peng et al. (2009) and Wu et al. (2005). Notably, dopaminergic neurons in rat midbrain slices were not as sensitive as the primary cultures, requiring ≥50 μM to elicit a significant decrease in dopaminergic neurons (Shimizu et al. 2003). However, Shimizu et al. (2003) did not report whether the neuron count was blinded, and in the absence of corroborating information from an independent study, OPP was less confident in the results presented in this study. Other rodent *in vitro* dopaminergic neuron models (e.g. the N27 cell line) were not tested at low enough concentrations to determine their relative sensitivity to the primary cultures and midbrain slices. An adequate study investigating human dopaminergic neuron viability was not available in the literature database; therefore, susceptibility of human dopaminergic neurons to paraquat exposure was inferred from the responses observed in human cell lines with dopaminergic characteristics or that are precursors to dopaminergic neurons. The most sensitive models, SH-SY5Y, MESC2.10, and hNPCs, exhibited a reduction in viability following exposure to paraquat concentrations ≥10 μM. (McCarthy et al. 2004; Ortiz-Ortiz et al. 2009; Huang et al. 2016; Chang et al. 2013; Dou et al. 2011; Ortiz-Ortiz et al. 2011).

Other PD-specific outcomes were less studied in the *in vitro* literature. In addition to its role as an important biomarker for assessing dopaminergic neuron health in *in vitro* and animal models, TH can also provide information on the capacity for catecholamine synthesis. TH levels either increased or were unaffected by paraquat exposure in the SH-SY5Y cell line (Caputi et al. 2015; Yang and Tiffany-Castiglioni 2005). Likewise, paraquat elicited an increase in TH activity in rat PC12 cells at 50 μM and did not have a significant impact on TH activity (estimated based on DOPA levels) in rat striatal tissue slices at concentrations up to 1000 μM (Hirata et al. 1986). Wu et al. (2005) investigated the impact of paraquat exposure on dopamine uptake. The authors observed a significant dose dependent decrease in uptake in rat primary mixed neuron-glial cultures following 6-7 day exposure to doses ≥0.5 μM . The decrease in dopamine uptake was concordant with qualitative and quantitative evidence of dopaminergic neuron degeneration and the authors suggested the two outcomes were related.

General Neurotoxicity Outcomes

The remaining outcomes assessed in the *in vitro* literature are non-specific forms of toxicity that are commonly associated with paraquat or neurological diseases such as PD. These general outcomes – reduced cell viability, increased apoptosis, oxidative stress, mitochondrial disruption, and inhibition of the ubiquitin-proteasome system – were studied extensively across a number of *in vitro* nervous system models. Although the outcomes are not specific to PD, the results are pertinent to this review because they may have a mechanistic connection to the disease (Baltazar et al. 2014; Terron et al. 2018).

Cell viability and cell death were assessed using several different assays. Each assay measured viability or death based on unique changes in the cell and lead to several inconsistencies when multiple assays were employed for the same cell line. Despite the inconsistencies, in general, rodent cell lines, particularly the rat primary neuron cultures, exhibited greater sensitivity to paraquat treatment compared to the human cell models. In addition to species, the source (e.g. region of the brain) and type of neuron population also dictated the response to exposure. Dopaminergic neurons in rat primary midbrain cultures, for example,

were one of the most vulnerable neuron populations to paraquat exposure (decreased viability at ≥2 µM after 24 hours of exposure; Klintworth et al. 2007), whereas the general midbrain neuron population from the same culture was tolerant of paraquat exposure up to 48 hours at concentrations <100 µM (Klintworth et al. 2007; Lopert et al. 2012). Neurons collected from the cerebral cortex of rat fetuses exhibited comparable sensitivity to dopaminergic neurons, with a dose dependent reduction in viability from exposure to ≥2 µM for at least 24 hours (Kim et al. 2004; Rathinam et al. 2012). Hippocampal neurons from 7-day-old rat pups, on the other hand, were less sensitive compared to dopaminergic and cortical neurons (reduced viability ≥10 µM within 48 hours) but more vulnerable than the general midbrain neuron population (Vornov et al. 1998). Selective degeneration of pyramidal neurons from the CA1 region was also observed in the hippocampal slices and was akin to the selective vulnerability of midbrain dopaminergic neurons (Vornov et al. 1998). The other rodent models examined were either not tested at a low enough concentration to compare sensitivity or were more tolerant of paraquat exposure compared to the primary neuron cultures.

Most of the human cell death/viability assays were conducted with SH-SY5Y cells and reported reduced viability and/or increased cell death after exposure to concentrations ≥10 µM (McCarthy et al. 2004; Ortiz-Ortiz et al. 2009). Decreased viability was also observed at concentrations ≥10 µM in hNPCs (Huang et al. 2016; Chang et al. 2013; Dou et al. 2011) and in both differentiated and undifferentiated MESC2.10 cells cultured from 8-week-old human embryonic ventral mesencephalic tissue (Ortiz-Ortiz et al. 2011). As mentioned previously, these data were used to infer the sensitivity of human dopaminergic neurons due to the lack of reliable *in vitro* data on human dopaminergic neuron cultures. Other human cell lines that were assessed for cell viability and death in the presence of paraquat were either not tested low enough to support sensitivity comparisons or were less sensitive to paraquat exposure compared to the SH-SY5Y, MESC2.10, and hNPCs.

Several studies investigated the mechanism of cell death alongside the cell viability assays. These studies consistently report a significant increase in apoptosis prior to or concurrent with the decline in cell viability. Klintworth et al. 2007 observed significant increases in apoptosis at concentrations ≥50 µM and ≥20 µM in undifferentiated and differentiated rat PC12 cells, respectively, whereas cell viability was significantly decreased at ≥50 µM regardless of differentiated status. Peng et al. (2004) noted evidence of apoptotic cell death in the rat dopaminergic N27 cell line (e.g. increased caspase-3 activity) that was concurrent with a substantial decrease in cell viability at 400 µM. Kim et al. (2004) reported morphological changes typical of neurons that have undergone apoptosis – shrunken body, condensed nuclei, and disintegrated dendrites – in the rat primary cortical cells treated at concentrations that decreased cell viability (≥10 µM paraquat). Evidence of apoptosis was also observed in mouse primary mesencephalic cultures at concentrations ≥25 µM that elicited a decline in dopaminergic neuron count (Choi et al. 2008; Choi et al. 2010). In the Choi et al. (2008) study, apoptosis increased in a dose dependent manner but was not significantly different from controls and apoptosis was inferred from a non-significant increase in caspase-3 activation in Choi et al. (2010). It is important to note, however, that the findings in both studies represent the response for the entire midbrain neuron culture and may have concealed a more pronounced effect in the vulnerable dopaminergic neuron subpopulation. Human *in vitro* models also initiated apoptosis in response to paraquat exposure. Evidence of apoptosis in the form of morphological changes and increased caspase-3 activity correlated with a decrease in SH-SY5Y cell viability at 10 µM (McCarthy et al. 2004). In human neural progenitor cells, evidence of apoptosis and a slight, but significant reduction in cell proliferation was observed at concentrations as low as 1 µM, which preceded a decline in cell viability (Chang et al. 2013).

Redox cycling leading to the production of reactive oxygen species (ROS) is recognized as a key cellular event in the paraquat mechanism of toxicity (Dinis-Oliveira et al. 2008). It comes to no surprise then that the *in vitro* literature reports evidence of oxidative stress in various human and rodent nervous system cell models. In fact, 71 of the *in vitro* studies identified as relevant for this systematic review used paraquat exclusively as a positive control to elicit oxidative stress. Given that the focus of these studies was not on paraquat toxicity *in vitro*, they were not included in the data evaluation. Studies with the stated goal of assessing paraquat toxicity *in vitro* consistently reported evidence of oxidative stress – increased ROS levels or signs of lipid peroxidation – in human and rodent models (including dopaminergic cell lines and primary rat mesencephalic cultures) at or below concentrations that caused a decrease in cell viability and survival (Peng et al. 2009; Chang et al. 2013; McCarthy et al. 2004; Dou et al. 2016; Case et al. 2016; Cristovao et al. 2009). Oxidative stress was observed as low as 0.5 μM (Peng et al. 2009; Wu et al. 2005) and 10 μM (Dou et al. 2016) in rodent and human *in vitro* models, respectively. The confluence of these outcomes suggests a causal relationship. Oxidative stress can be induced via direct contact with paraquat; however, several studies indicate extrinsic factors are also involved. Peng et al. (2009) and Wu et al. (2005), for example, note that dopaminergic neurons were more tolerant of paraquat exposure in microglia-depleted cultures suggesting that microglia, which produce ROS in the presence of paraquat, were responsible for the degeneration of dopaminergic neuron in primary rat mesencephalic cultures. Analyzing antioxidant levels also provides clues as to the tolerance of a given model system to oxidative stress. Significant decreases in antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) were observed following paraquat exposure at 100 μM in human neural progenitor (Dou et al. 2016) and were secondary to clear evidence of lipid peroxidation and cytotoxicity. That lipid peroxidation and cytotoxicity were observed at lower paraquat concentrations in the human neural progenitor cells suggests that the antioxidant mechanisms were overwhelmed at those concentrations despite maintaining basal levels. Two other antioxidant enzymes, glutathione peroxidase (GPx) and glutathione-S-transferase (GST), were observed to decrease and increase, respectively, in SH-SY5Y cells during a 48-hour exposure to 500 μM that also elicited a decrease in cell viability (Yang and Tiffany-Castiglioni 2005). Another antioxidant enzyme, glutathione reductase (GR), was not affected (Yang and Tiffany-Castiglioni 2005). Glutathione (GSH) levels were also assessed in several cell lines to evaluate oxidative stress. Depletion of GSH was noted after 24 hours of exposure to ≥10 μM in rat hippocampal slices and preceded neuronal injury (Vornov et al. 1998). A similar response was observed in SH-SY5Y cells after 48 hours of exposure to ≥10 μM (McCarthy et al. 2004; Yang and Tiffany-Castiglioni 2005). Collectively, these data demonstrate that paraquat exposure can induce oxidative stress in human and rodent cell lines with dopaminergic characteristics as well as rodent primary neuron cultures. Furthermore, the fact that oxidative stress coincides with cytotoxicity suggests that it plays a role in the decline in cell viability and survival.

At the subcellular level, paraquat targets the mitochondria, eliciting a series of effects that can disrupt its ability to function, affect structural integrity, and promote apoptosis (Baltazar et al. 2014). Inhibition of NADH-ubiquinone reductase (Complex I), thought to be the initiating event in the cascade of effects that lead to mitochondrial dysfunction (Cocheme and Murphy 2008) and, ultimately, PD (Terron et al. 2017), is reported in both human and rodent *in vitro* nervous system models. Complex I inhibition was reported in SH-SY5Y human neuroblastoma cells and was accompanied by a reduction in mitochondria membrane potential and ATP production at 500 μM (Yang and Tiffany-Castiglioni 2008). Paraquat was observed to elicit a reduction in membrane potential and ATP production, and/or an increase in mitochondrial ROS at concentrations as low as 10 μM and 2 μM in human (SH-SY5Y cells) and rodent (primary cortical neurons, PC12, and NG108-15 cells) *in vitro* models, respectively (Ding and Keller 2001; McCarthy et al. 2004; Yang and Tiffany-Castiglioni 2005; Yang and Tiffany-Castiglioni 2007; Chau et al. 2010; Huang et

al. 2012; Case et al. 2016); however, these findings could not be correlated to Complex I activity because it was not measured in these studies. Paraquat exposure also significantly increased mitochondrial fragmentation in SH-SY5Y cells at concentrations ≥125 μM (Zhao et al. 2017) and reduced Complex V activity in the same cell line at 500 μM (Yang and Tiffany-Castiglioni 2007) – the same concentration observed to inhibit Complex I. Morphological changes, reduced membrane potential, and increased mitochondrial superoxide production were also noted in primary rat cortical neurons at concentrations as low as 2 μM (Zhao et al. 2017). In general, observations of mitochondrial dysfunction correlated with the decline in cell viability. Though this does suggest a mechanistic connection, it should be noted that many of the *in vitro* studies assessed cell viability based on metabolic activity (MTT or MTS assays) and would thus be sensitive to changes in mitochondrial function.

Altered protein ubiquitination, proteasome activity, and autophagy is also reported at cytotoxic paraquat concentrations in rat and human nervous system *in vitro* models. The ubiquitin-proteasome system (UPS) and autophagy are responsible for breaking down misfolded or damaged proteins within the cell (Navarro-Yepes et al. 2016) and is of particular importance to PD because it is involved in regulation of α-synuclein (Branco et al. 2010). A duration and concentration-dependent reduction in 20S proteasome activity in SH-SY5Y human neuroblastoma cell lysates was observed within 6 hours of exposure to concentrations ≥20 μM (Ding and Keller 2001). The reduction in proteasome activity preceded cell death which was not observed until 12-24 hours of exposure (Ding and Keller 2001). Interestingly, at lower concentrations proteasome activity increased in the lysates from the same cell line (McCarthy et al. 2004). Navarro-Yepes et al. (2016) noted that the apparent impact of paraquat on proteasomal activity in SK-N-MC cells differed based on whether cell or cell lysates were assayed. The authors observed a concentration-dependent increase in proteasome activity at concentrations ≥100 μM in intact SK-N-MC cells, whereas proteasome activity was significantly decreased in cell lysates at concentrations ≥500 μM. Based on these results, the authors posited that impairment of proteasomal activity is associated with the loss of cell viability. The same authors report that paraquat at concentrations ≥500 μM deplete the ubiquitin protein pool, decrease protein ubiquitination, and impair autophagy flux (Navarro-Yepes et al. 2016). These findings suggest that autophagy, not the proteasome, regulate paraquat induced SK-N-MC cell death (Navarro-Yepes et al. 2016). Conversely, the level of ubiquitinated proteins increased in SH-SY5Y cells at 500 μM and were concurrent with a decrease in proteasome activity (Yang and Tiffany-Castiglioni 2007) indicating that the mechanism of UPS and autophagy inhibition may be cell line dependent. The response in the rat N27 cell line was analogous to the observations in SH-SY5Y cells. Inhibition of 20S proteasomal activity was observed after exposure to 500 μM for 48 hours (Chinta et al. 2008).

The *in vitro* outcomes discussed above were the most relevant to the weight of evidence analysis, but they were not the only outcomes reported in the *in vitro* literature. A number of studies explored molecular, genetic, and biochemical outcomes to elucidate the mechanisms underlying the cellular and subcellular responses. These data, while important, were considered more useful for development of an AOP, which is beyond the scope of this systematic review.

8.0 Weight of Evidence Analysis

The weight of evidence analysis considered all relevant data from the human, animal, and *in vitro* evidence streams to evaluate the association between environmentally relevant paraquat exposure and PD. Integration of these three evidence streams was performed in accordance with the methods outlined in the OPP Epidemiology Framework. Per the framework, the weight of evidence was analyzed based on the modified Bradford Hill Criteria (Hill 1965) which includes considerations for dose response, temporal

concordance, strength, consistency, coherence, specificity, and biological plausibility. These considerations and the uncertainties in the weight of evidence analysis are discussed below.

Dose Response and Temporal Concordance

Establishing a dose response and temporal concordance requires mechanistic knowledge of the key events leading to the outcome of interest. In the case of PD, the pathology is well described, but the etiology is not as well understood, in part because it is thought to be multifactorial (Antony et al. 2013). Exposure to environmental contaminants, such as paraquat, is proposed as one of several risk factors in the development of PD (Allen et al. 2013), but the connection between exposure and effects remains elusive. Baltazar et al. (2014) describe five proposed mechanistic pathways by which paraquat exposure could elicit PD in humans: induction of oxidative stress and inflammation, mitochondrial dysfunction, apoptosis and autophagy, inhibition of the ubiquitin-proteasome system (UPS), and induction of synucleiopathy and tauopathy. The *in vitro* literature is exhaustive in its efforts to delineate these proposed pathways in relevant model systems.

Evidence of paraquat-induced oxidative stress, mitochondrial dysfunction, apoptosis, impairment of autophagy, α-synuclein aggregation, and inhibition of the ubiquitin-proteasome system was observed in rodent dopaminergic neurons, primary rodent neuron cultures, human neural progenitor cells, and/or rodent and human dopaminergic cell lines. With few exceptions, there was an apparent dose and temporal concordance between the mechanistic outcomes and declining cell viability/survival. Dopaminergic neurons from primary midbrain cultures, one of the most vulnerable neuron populations assessed and a selective target of paraquat (Klintworth et al. 2007), exhibited a decline in number that was concordant with an increase in oxidative stress produced by neighboring microglia and an increase in apoptosis and apoptotic enzymes for the whole culture (Peng et al. 2009 and Wu et al. 2005). Oxidative stress and apoptosis were also observed in dopaminergic rodent and human cell lines (Peng et al. 2009; Chang et al. 2013; McCarthy et al. 2004; Dou et al. 2016; Case et al. 2016; Cristovao et al. 2009) along with evidence of mitochondrial dysfunction (Yang and Tiffany-Castiglioni 2008; Ding and Keller 2001; McCarthy et al. 2004; Yang and Tiffany-Castiglioni 2005; Yang and Tiffany-Castiglioni 2007; Chau et al. 2010; Huang et al. 2012; Case et al. 2016; Zhao et al. 2017) at or below concentrations that elicited a reduction in cell viability and/or increase in necrotic cell death. Inhibition of the UPS was noted in both rodent and human dopaminergic cell lines as well (Ding and Keller 2001; Yang and Tiffany-Castiglioni 2007; Chinta et al. 2008); however, it was only observed prior to loss of cell viability in the SH-SY5Y human cell line. Impairment of autophagy preceded loss of cell viability in SK-N-MC human cell line and was proposed as the cause of cell death in this cell line rather than UPS inhibition (Navarro-Yepes et al. 2016). Observation of these events preceding or coinciding with the decline in cell viability/survival in dopaminergic neurons and cell lines with dopaminergic characteristics suggest these outcomes are connected to dopaminergic neuron degeneration, one of the principle PD hallmarks. A similar sequence of events leading to loss of cell viability was observed in cortical neurons and hippocampal tissue slices indicating that paraquat could elicit neuron degeneration in other regions of the brain via a similar mechanism.

In vitro outcomes related to other biochemical and cellular PD hallmarks (e.g. Lewy body formation and neurochemical disruption) were not studied as comprehensively across the *in vitro* models. Paraquat was observed to accelerate fibril formation in recombinant α-synuclein preparations (Manning-Bog et al. 2001; Anselmi et al. 2018) and α-synuclein levels increased in SH-SY5Y cells subsequent to decreased proteasome activity (Caputi et al. 2015). This sequence of events is consistent with the known regulatory mechanisms that connect the UPS and α-synuclein formation and indicate that paraquat can elicit subcellular changes that could manifest in Lewy body formation in dopaminergic cells; however, it was

not possible to discern if a similar pattern occurred in other *in vitro* models including primary neuron cultures due to a lack of data for one or more outcomes involved. A larger dataset was available to assess TH expression and/or activity across *in vitro* models. In a human cell line with dopaminergic characteristics, TH protein levels either increased or remained constant at exposure levels that elicited a reduction in cell viability (Caputi et al. 2015; Yang and Tiffany-Castiglioni 2005). Likewise, no change in TH activity was observed in rat striatal tissue exposed to paraquat though the status of the tissue viability was not reported (Hirata et al. 1986). In contrast, TH protein levels in rodent primary mesencephalic cultures – estimated based on TH immunoreactivity – decreased concurrent with the decline in dopaminergic neuron health (Wu et al. 2005; Peng et al. 2009; Klintworth et al. 2007). Dopamine uptake and dopaminergic cell viability were also correlated in rat mixed neuron-glial cultures (Wu et al. 2005). It is clear from these data that dopamine production (based on TH levels) and uptake correspond to the health of dopaminergic neurons but the sequence of the outcomes (e.g. whether neurochemical disruption precede cell degeneration) is not evident.

Mechanistic pathways were explored in only a few of the acceptable animal studies evaluated for the PD systematic review. Two studies (Ren et al. 2006 and Satpute et al. 2017) observed general toxicity (oxidative stress, inflammation, and mitochondrial dysfunction) in brain tissue at similar doses that elicited PD-like outcomes. Naudet et al. (2016) reported evidence of neuroinflammation (changes in astrocyte activity) in the enteric nervous system but the reviewer could not could not evaluate the connection between this finding and other PD hallmarks evaluated in the study given deficiencies in the methods and reporting for the motor coordination assessment. The remaining animal studies examined apical PD-like outcomes only and did not investigate mechanistic endpoints. Large gaps in the dose and temporal relationship created by variation in study design, inconsistencies in the parameters assessed, and a paucity of data precluded evaluation of the dose and temporal concordance for mechanistic endpoints across studies. Although not conclusive evidence, that oxidative stress, inflammation, and mitochondrial dysfunction were observed at dose levels that also elicited PD-like hallmarks in the aforementioned studies suggests a mechanistic connection to PD, as proposed in Baltazar et al. 2014, cannot be ruled out.

Dose and temporal relationships for the PD hallmarks were also difficult to evaluate in animal models due to the small number of studies presenting consistent and reliable positive results, variability in the outcome assessment methods and types of outcomes reported, and the limited dose range and exposure duration investigated across these studies. Two of the four animal studies reporting positive results only tested paraquat at a single dose level (either 7.2 or 14.5 mg ion/kg) precluding evaluation of the dose-response relationship for the outcome(s) within the study. Moreover, all four studies examined the apical outcomes of interest at a single time point only. In combination, the four studies reporting positive results (Ren et al. 2006; Satpute et al. 2017; Lou et al. 2016; Endo et al. 1988) suggest that PD-like motor impairment (loss of motor coordination and reduced motor activity) occur in male mice after exposure for at least 4 weeks to oral doses ≥7.2 mg/kg/day (≥10 mg dichloride/kg/day). Disruption of the nigrostriatal pathway catalyzed by dopaminergic neuron degeneration and subsequent depletion of striatal dopamine is thought to be the underlying cause of the motor deficits (Dauer and Przedborski 2003; Anthony et al. 2013) and disruption in other regions of the brain are hypothesized as a contributing factor in the manifestation of non-motor symptoms (Baltazar et al. 2014); therefore, a progression from neuropathological and neurochemical effects to neurobehavioral effects with increasing dose and duration would be expected. Significant neurochemical changes in the SN (decreased DA) were observed earlier (within 3 days) at higher doses (30 mg/kg/day; Endo et al. 1988); however, there are no other studies that bridge the gap between this early onset effect and the apical PD outcomes. Moreover, these early onset findings were attributed to a 24% w/w formulation rather than paraquat technical or high purity product. Of the subchronic studies, Ren et al. (2006) was the only one to confirm that decreased striatal dopamine

levels and decreased TH immunoreactivity in the SN accompanied motor impairment. Neither Satpute et al. (2017) nor Lou et al. (2016) investigated whether changes in neurochemistry and/or neuropathology contributed to the motor deficits observed over shorter exposure duration at the same or higher dose levels. The lack of empirical support for the neuropathological and neurochemical findings observed at a single dose level in Ren et al. (2006) lowered confidence in those outcome results. Furthermore, the paucity of neuropathological and neurochemical outcome data for oral exposure in mice precluded evaluation of the dose and temporal connection between PD-like biochemical, cellular, and tissue level changes and the observed motor impairment.

The dose and temporal relationship of the PD-like hallmarks discussed up to this point was limited to data for oral exposure in mice. Rats, in general, exhibited few characteristic PD-like effects following exposure, though most studies were conducted at dose levels lower than those explored in mice. In contrast with the mouse data, the highest repeat dietary dose, ~11 mg/kg/day, investigated in rats elicited no evidence of behavioral or neuropathological abnormalities. These findings do not necessarily dispute the pattern observed in male mice as rats may be less sensitive to paraquat exposure; however, this assumption could not be confirmed due to a lack of data on PD-like effects in rats at higher repeat doses. The risk assessment relevant animal literature database also lacked sufficient reliable data to evaluate dose and temporal concordance for dermal and inhalation exposure. Overall, the data reported in the risk assessment relevant animal studies were not sufficient to establish dose and temporal concordance for the PD-like hallmarks.

The available epidemiologic studies on paraquat exposure and PD had limited ability to assess dose-response and temporal concordance. With respect to dose-response, all epidemiologic studies relied on indirect exposure methods to assign categorical levels of exposure using either survey questionnaires or spatial information on agricultural pesticide use and land use in relation to residential/occupational address. It is possible to assess dose-response using categorical levels of exposure, but none of the studies evaluated the dose-response relationship using formal statistical methods (e.g., p-trend or other categorical data analysis methods). In many studies, this is because investigators enrolled too few study subjects reporting paraquat exposure (e.g., Firestone et al., 2005 and 2010) or were only able to ascertain ever/never exposure to paraquat (e.g., Kamel et al., 2007 and Tanner et al., 2009).

The FAME study, nested with AHS and reported by Tanner at al. (2011), collected questionnaire information on cumulative lifetime use of paraquat, but only assessed stratified their analysis of exposure by median lifetime days. Specifically, Tanner et al. (2011) reported the OR increase from 2.4 (95% CI: 1.0-5.5, n= 10 exposed cases) in individuals who reported ≤ median duration of 8 lifetime days of paraquat use to 3.6 (95% CI: 1.6-8.1, n= 13 exposed cases) in individuals reporting > median lifetime days of paraquat use. However, this does not constitute a formal analysis of the dose-response because the two exposure categories were not compared. Moreover, the number of exposed individuals in each category was relatively small and no rationale was provided for using the median of 8 lifetime days of paraquat use as a cut-point for making comparisons. This latter consideration is relevant because it is unclear that 8 lifetime days of exposure is biologically meaningful in terms of the magnitude and frequency of exposure. Similarly, Liou et al. (1997) used a questionnaire to ascertain years of paraquat use and reported no evidence of an association in the 1-19 years of paraquat use category (OR = 0.96, 95% CI: 0.24-3.83, n = 7 exposed cases) but evidence of a strong positive association in the ≥20 years paraquat use category (OR = 6.44, 95% CI: 2.41-17.2, n = 24 exposed cases. However, there were only a limited number of subjects reporting 1-19 years of paraquat use and no consideration of differences in the number of exposure-days per year.

The available epidemiologic studies also have limited ability to evaluate the temporal relationship between paraquat exposure and PD. The primary reason for this is that PD has a long latency period and is difficult to prospectively investigate in study populations that may be exposed to paraquat. As such, the majority of studies used case-control designs and retrospectively assessed past paraquat exposure. This approach may introduce recall bias, particularly when PD cases and controls are asked to report on past use of paraquat during their lifetime, and the studies that instead used GIS-based methods have not been validated and may lack specificity with respect to estimate long-term paraquat exposure.

Strength, Consistency, and Specificity

Consistency of disease-related outcomes across species and biological levels as well as the strength and specificity of association between a chemical and the outcomes strengthens the argument for causality between exposure and disease.

The available evidence from epidemiologic studies was mixed with regard to strength and consistency of reported findings on the association between paraquat exposure and PD. Specificity was not fully evaluated and is considered less relevant with respect to the epidemiology studies because a complex range of genetic, behavioral, and environmental factors may contribute to progression of PD. In the FAME study nested within AHS, Tanner et al. (2011) reported a moderately strong positive association for ever use of paraquat (OR = 2.5, 95% CI, 1.4-4.7), based on 23 paraquat exposed cases. However, this reported finding is based on analysis of incident and prevalent cases in the AHS cohort combined and is difficult to interpret in relation to the findings reporting Kamel et al. (2007), which included many of the same PD cases as the FAME study. Notably, Kamel et al. (2007) did stratify their analysis by incident and prevalent PD cases and reported no evidence of a significant positive association with prevalent PD (OR = 1.8; 95% CI, 1.0-3.4, n = 14 paraquat exposed cases) and no evidence of an association with incident PD (OR = 1.0; 95% CI, 0.5-1.9, n = 11 paraquat exposed cases). The two other agricultural study populations identified included the French AGRICAN cohort (Pouchieu et al., 2018) and Washington State Department of Public Health study population (Engel et al., 2001). Pouchieu et al. (2018) reported evidence of a positive association in a cross-section study of the French AGRICAN cohort. These studies were both determined to be of low quality, however, and contributed less weight-of-evidence in the agency assessment of the available epidemiologic literature.

Eight hospital-based studies examined potential occupational paraquat exposure and PD. Five of these studies had only a small number of cases and contributed limited weight in the agency's assessment (Firestone et al. 2005 and 2010; Dhillon et al., 2008; and Hertzman et al., 1990 and 1994). Results of the remaining three studies, all rated moderate, are mixed and may be subject to recall bias, limitations in their exposure assessment approach, and potential selection bias. Liou et al (1997) reported the strongest positive association, based on individuals reporting ≥20 years of paraquat use in Taiwan (OR = 6.44, 95% CI: 2.41-17.2, n = 24 exposed cases). A similar association was observed for use of herbicides/pesticides more generally in the Liou et al. (1997) study, however, so it is unclear if the association is directly attributable to paraquat use, overall pesticide use more broadly, or another confounding factor correlated with reporting pesticide use. Tanner et al. (2009) also reported a non-significant positive association in their multicenter PD study (OR = 2.80, 95% CI: 0.81-9.72). However, this reported association was based on only 9 exposed cases and was also similar to the reported associations for both other specific pesticides and pesticide use more generally. In contrast, Van der Mark et al. (2014) reported no association between occupational paraquat exposure and PD, based on self-reported crop activities and crop-exposure matrix. The results of two additional studies on non-occupational paraquat exposure were also mixed, with the PEG study reporting no evidence of an association in analysis that focused specifically on paraquat only (Costello et al., 2009) and Brouwer et al. (2017) in their case-control study in the Netherlands.

The animal database consisted of 11 acceptable risk assessment relevant studies and explored PD hallmarks and general neurotoxicity in both mice and rats. In general, the animal results were mixed across the rodent models examined. Positive results for PD-like hallmarks were reported sporadically with all findings observed in mice. In the mouse model, males were the only sex to exhibit PD-like hallmarks following oral exposure (Ren et al. 2009; Satpute et al. 2017; Lou et al. 2016; Endo et al. 1988) and these effects were observed across three mouse strains. Only one acceptable study included evaluation of female mice. The study reported neuroinflammation in the enteric nervous system which has a hypothesized mechanistic connection to PD but is not itself a principle hallmark of PD (Naudet et al. 2016). No other reliable female mouse outcome information was available to evaluate the consistency in the response to oral treatment between sexes. The studies with positive findings in male mice generally reported large magnitude of change (>40%) from control response for the outcomes assessed suggesting they were not an artifact of variability within the population tested. With the exception of the neurochemical results in Endo et al. (1988), the study design and description of the paraquat product was sufficient to specifically link the positive findings to paraquat exposure. Endo et al. (1988) used an end-use product, Gramoxon; therefore, contribution to the reported outcomes from other ingredients in the end-use product cannot be ruled out. In the rat model, no evidence of a PD-like response was observed following oral exposure. These studies used either a technical product (33.4% w/w paraquat ion) or a product at a purity similar to the technical formulation (33% paraquat ion w/w). The products tested in these studies likely contain additional components that would not be found in solutions made from the high purity paraquat products and thus less specificity compared to the mouse studies. Nevertheless, the outcome results do have specificity to paraquat products currently registered in the US. The strength of an association was not relevant to studies exclusively reporting null results. Overall, the positive findings in male mice present a strong and specific association to oral paraquat exposure but the findings lack consistency across rodent species and there was not enough data to evaluate consistency across sexes. It should be noted that the mixed findings may be, in part, related to the limited number of risk assessment relevant acceptable animal studies available for evaluation.

Only a single, acceptable study was available to assess PD-like outcomes from intranasal/inhalation exposure and no acceptable dermal studies were available. Although the intranasal study reported similar null results between mice and rats, the exposure levels were not equivalent, not all outcomes were assessed in both species, confidence in outcome results varied between species, and only males were tested. Given these constraints, consistency in the response from intranasal exposure could not be evaluated from this single study. The paraquat product used by the authors was assumed to be high purity as deduced from the source; however, deficiencies in the methodology lower confidence in the reported lack of association between intranasal paraquat exposure and PD-like hallmarks.

The *in vitro* database consisted of 244 studies that were considered relevant to evaluating the association between paraquat exposure and PD. These *in vitro* studies were parsed for outcomes that would be the most useful to connect cellular and subcellular effects to PD hallmarks in the animal and human studies. Of the 244 studies, 34 were considered for the weight of evidence analysis because they represented the most sensitive response to the outcomes of interest in rodent and human models. Relevant outcomes were reported in numerous human and rodent *in vitro* nervous system models including cell lines with dopaminergic properties and primary neuron cultures. Consistency across these *in vitro* models was not expected given differences in origin and cell type; however, for a few outcomes, particularly cell viability, the same *in vitro* model produced different results across studies. This was most notable in the SH-SY5Y cell line and suggested variability in sensitivity. These mixed findings may be related to differences in assay methodology, culturing technique, differentiation status, or source of the *in vitro* model, but still impact confidence in these *in vitro* results. All *in vitro* outcomes evaluated were qualitatively, but not

quantitatively similar across rodent and human *in vitro* models. For all outcomes, rodent models exhibited greater sensitivity to paraquat exposure compared to human models. It should be noted, however, that not all outcomes were examined in each model, human and rodent *in vitro* models were not always tested at the same concentrations, and a majority of the human *in vitro* data were derived from neural progenitor cells and cell lines that exhibit dopaminergic features rather than primary neuron cultures. The *in vitro* studies considered in the weight of evidence analysis tested paraquat products that were >98% purity (either explicitly reported by the authors or deduced based on the source of the product) indicating that the effects observed could be attributed to paraquat alone. Most *in vitro* studies presented data graphically without numerical values either in the text or in a table. Consequently, the strength of the association for the various reported outcomes could not be evaluated for the *in vitro* body of evidence.

Coherence and Biological Plausibility

Coherence of outcomes across lines of evidence lends additional weight to the presumption of a causal association between the chemical and disease. There is, however, weak evidence of coherence across the three evidence streams evaluated for this systematic review. *In vitro* data demonstrate that direct cellular contact with paraquat results in a series of subcellular effects that precede or coincide with a reduction in cell viability and ultimately, cell death. In rodent midbrain cultures, dopaminergic neurons are selectively targeted by paraquat and experience significant deterioration, one of the principle PD hallmarks, that is exacerbated following prolonged exposure (Peng et al. 2009; Wu et al. 2005; Klintworth et al 2007). Sensitivity to cellular degeneration was also observed in rodent neurons from other brain regions including hippocampal tissues (Vornov et al. 1998) and primary cortical neurons (Kim et al. 2004; Rathinam et al. 2012). Human *in vitro* models including cell lines with dopaminergic features were more tolerant of paraquat exposure than the most sensitive rodent models, yet the subcellular and cellular response to paraquat treatment were qualitatively similar. Despite evidence of subcellular and cellular toxicity *in vitro* across species, PD-like hallmarks were not prevalent in the animal literature evaluated for this systematic review and few epidemiology studies provided evidence of an association between PD and paraquat exposure.

Few animal studies exhibited coherence with the subcellular and cellular outcomes described in the *in vitro* literature. Of the animal studies evaluated, Ren et al. (2009) was the most effective at illustrating coherence between biochemical, subcellular, cellular, and organismal effects in animals exhibiting PD-like symptoms. Ren et al. (2009) observed oxidative stress in brain tissues at the same dose level that elicited a suite of PD-like effects including decreased motor activity, decreased DA levels, and decreased TH immunoreactivity in the SN. Satpute et al. (2017) reported oxidative stress, general inflammation and mitochondrial dysfunction in brain tissues that was concurrent with a loss of motor coordination. The authors successfully bridged general and PD-like outcomes across levels of biological organization; however, unlike Ren et al. (2009), the study lacked data to connect the subcellular and organismal effects to cellular degeneration that is a principle hallmark of PD. These studies demonstrate that outcomes reported in the *in vitro* literature are also observed in whole animal models at doses that elicit PD-like effects. Naudet et al. (2016) and Endo et al. (1988) also observed changes at the subcellular level (increased astrocyte activity and neurochemical changes, respectively) similar to responses observed in the *in vitro* literature, but limitations in the methodology and a lack of additional outcome information, respectively, precluded connecting these results to PD-like outcomes at higher biological levels. The remaining animal studies did not exhibit coherence with the *in vitro* evidence because they either reported null results (Brammer 2006; Chivers 2006; Rojo et al. 2007) and/or results contrary to the *in vitro* findings (e.g. increase in striatal DA rather than decrease; Widdowson et al.1996), methodology or

deficiencies limited outcome reporting to toxicokinetic information (Prasad et al. 2007; Minnema et al. 2014), or the study focused on whole animal outcomes only (Lou et al. 2016).

Coherence between the laboratory and epidemiology data was similarly tenuous. PD diagnosis in the epidemiology studies was either based on a doctor examination or was self-reported. All participants were alive at the time of diagnosis and the diagnosis was based on behavioral symptoms. Behavioral changes – loss of motor coordination and reduced motor activity – observed in the animal studies following paraquat exposure were qualitatively similar to the behavioral symptoms that were noted in the diagnosis of PD in the epidemiology studies that reported a positive association. It was, however, not possible to compare the data quantitatively because the epidemiology studies relied on either survey questionnaires or spatial information on agricultural pesticide use and land use in relation to residential/occupational address to indirectly estimate exposure in the participants. Biochemical, subcellular, cellular, and tissue level effects reported in the animal and *in vitro* literature were not assessed in the epidemiology literature. Consequently, coherence between laboratory and epidemiology evidence could not be assessed for those outcomes.

Biological plausibility is the final consideration in the weight of evidence analysis and evaluates the likelihood that the mechanisms and outcomes described in the laboratory studies will occur in humans. Regardless of the subcellular mechanism, the *in vitro* literature demonstrates that both rodent and human *in vitro* models are susceptible to direct paraquat exposure. Rodent *in vitro* models tended to be more sensitive to paraquat compared to human *in vitro* models; however, the outcomes observed in both models were qualitatively similar. Of the *in vitro* outcomes assessed, evidence of degeneration in both human and rodent dopaminergic neurons provided the best indication that paraquat exposure could elicit a cellular level effect in humans that is associated with PD and is thought to be the underlying cause of other PD hallmarks. Based on the rodent *in vitro* data, significant degeneration of dopaminergic neurons is expected to occur if paraquat levels are sustained at $\geq$0.5 µM for at least 6-7 days, $\geq$1 µM for at least 5 days, or at $\geq$2 µM for at least 24 hours in the midbrain. Rodent *in vivo* toxicokinetic data confirm that paraquat is distributed to the brain and can reach the midbrain following oral exposure; however, evidence of dopaminergic neuron degeneration in whole animal models was limited to a single finding of reduced TH optical density in a study that tested only at one dose level. The suite of PD-like hallmarks observed in these animals suggest the reduction in TH was related to dopaminergic neuron loss but the lack of empirical support for this finding at the same or other dose levels leaves open uncertainty as to whether oral exposure can achieve levels in the midbrain tissue that are cytotoxic. A validated PBPK model was not available to estimate brain tissue concentration at the dose levels assessed in the *in vivo* studies to address this uncertainty. Human dopaminergic neurons, on the other hand, are ostensibly more tolerant of paraquat exposure based on the responses in human cell lines with dopaminergic characteristics; however, given that these are immortalized cell lines and not primary cell/tissue cultures, they may not be an accurate reflection of dopaminergic neuron sensitivity in humans. The lack of a PBPK model also precluded assessment of interspecies differences in toxicokinetics. The available *in vitro* data thus demonstrate a qualitative similarity in dopaminergic neuron degeneration between rodents and humans, but there was not enough information to relate the findings in the *in vitro* models to the laboratory animal results nor to evaluate the biological plausibility of these *in vitro* findings for human exposure.

Biological plausibility of the laboratory animal findings was evaluated based on the likelihood that humans would be exposed to levels reported to elicit PD-like hallmarks in animals. Comparing the dose levels reported in laboratory animals to exposure in the human studies proved difficult, however, as the human studies estimated paraquat exposure using indirect approaches (e.g. questionnaires on paraquat use

history or GIS-based approaches). Exposure estimates calculated for the Registration Review risk assessment (D430827 W. Britton 2019) provided a more quantitative point of comparison for evaluating biological plausibility. Note that these estimates only pertain to exposure from products currently registered in the United States. Worst-case dermal (0.323 mg ion/kg/day for mechanically-pressurized handgun pastureland application with double-layer clothing and gloves) and inhalation (0.00125 mg ion/kg/day for liquid ground boom mixer/loader with engineering controls) exposure estimates for occupational activities conducted in accordance with labels on registered paraquat products are between 2 and 4 orders of magnitude below the lowest dose (7.2 mg ion/kg/day) that elicited PD-like hallmarks in whole animal models[12]. Likewise, worst-case chronic dietary exposure (0.00125 mg/kg/day in children 1-2 years old) is estimated to be close to 4 orders of magnitude below the oral dose level that was observed to elicit PD-like effects in mice. Based on these estimates, human exposure via the diet and/or in the field resulting from label-directed use of registered products is not likely to reach levels that elicited the neurodegenerative hallmarks of PD observed in laboratory animals.

Uncertainties

Data evaluation and the weight of evidence analysis was hindered by several uncertainties in the literature database compiled for the PD systematic review. These uncertainties were introduced as a result of limitations in data access, data gaps in the literature database, or methodology decisions.

Raw data were not available for the human and *in vitro* studies and, with four exceptions, the risk assessment relevant animal studies. Quality assessment and data evaluation for these studies relied on the information reported in the publication and assumptions in the absence of raw data. Without raw data, OPP was not able to perform its own statistical analysis nor re-evaluate the empirical results based on current policies and practices. As a result, uncertainties in the methods, data, and conclusions could not be resolved. Access to the full dataset allowed for a more thorough and independent review of the four risk assessment relevant studies that made the datasets available and OPP was more confident in the quality assessment and data evaluation for these studies.

The lack of data on PD-like outcomes in whole animal models following chronic exposure lead to some uncertainty in characterizing the impact of long-term exposure on nervous tissues. Long-term monitoring of neurobehavioral, neurochemical, and neuropathological outcomes is of particular importance to diagnosing a progressive neurodegenerative disease such as PD. Toxicokinetic data suggest long-term exposure is likely to result in prolonged contact between paraquat and brain tissues that will persist for a period of time after the exposure ends. The impact of long-term exposure on human health can be gauged from the available epidemiology data; however, chronic whole animal studies in a controlled laboratory setting provide greater specificity, and more robust dose and temporal concordance information for evaluating the consequences of long-term paraquat exposure. Although a chronic whole animal study designed to examine specific PD-like outcomes was not available, chronic guideline studies in rodents and non-rodents from the guideline registration database do provide some insight into the health of the nervous system following long-term exposure. None of these studies report evidence of unusual behavioral signs or abnormal histopathology in brain tissues of rats, mice, or dogs at sublethal chronic doses.

[12]The lowest oral dose was extrapolated to a dermal dose (144 mg ion/kg/day) using a dermal absorption factor of 0.3%, and corrected for low oral absorption (6%). The dermal extrapolation assumed the skin was intact; however, it should be noted that repeated exposure to paraquat can be corrosive to skin which would increase dermal absorption. Extrapolation of the lowest oral dose to an inhaled dose relied on the assumption that oral and inhalation absorption were equivalent.

The consequences of dermal and inhalation exposure were also not adequately explored in the animal literature. Dermal and inhalation are the principle routes of exposure for occupational uses, yet only one study was available to evaluate the PD-like outcomes for intranasal/inhalation exposure and no acceptable studies were available to evaluate dermal exposure. The weight of evidence analysis, therefore, relied heavily on data from oral studies. Toxicokinetic data suggest absorption is similar to or lower than oral exposure; however, a more robust dataset would increase confidence in the evaluation for these critical routes of exposure.

Although not required to form a conclusion on the association between paraquat exposure and PD, a validated PBPK model would address some of the uncertainty in the biological plausibility evaluation. Toxicokinetic data were available for rodents; however, there was not enough information to determine whether tissue concentration in the midbrain would be sustained at levels that were observed to elicit dopaminergic neuron degeneration *in vitro*. The tissue concentration estimates from a PBPK model would thus be useful for reconciling the findings in the laboratory animal studies with the *in vitro* data. A PBPK model would also support interspecies toxicokinetic comparisons to further evaluate the human relevance of the effects observed in laboratory animals.

Several important challenges were identified in the epidemiologic literature that introduced uncertainty in the evaluation of the relationship between paraquat exposure and PD. Importantly, PD progresses over a long latency period and is relatively rare in the general population. This makes it difficult to conduct prospective studies that can fully evaluate the temporal relationship between paraquat exposure and PD. For this reason, most of the epidemiologic studies identified used case-control design to identify a sufficient number of PD cases and retrospectively assess paraquat exposure. In these studies, exposure was assessed using questionnaires (never/ever use or cumulative lifetime days of use) on paraquat use history or GIS-based approaches. Assessment of exposure using a questionnaire does not provide a direct measure of exposure and may introduce recall bias if PD cases recall past paraquat use differently. Similarly, two studies used GIS-based approaches based on land-use data and residential/workplace address. This approach is not subject to recall bias but has not been fully validated to demonstrate that it can provide a reliable estimate of individual exposure. Regardless of study design, exposure assessment will remain a challenge in the assessment of PD because it is difficult to estimate chronic exposure and evaluate paraquat exposure in isolation of other pesticide and factors that may be associated with rural living.

9.0 Implications for Registration Review Human Health Risk Assessment

The PD systematic review was conducted to support the evaluation of human health risks for currently registered paraquat products. Given the abundance of studies on PD and PD-like effects in the paraquat literature, a systematic review of the literature was warranted to adequately characterize the impact of paraquat exposure on the nervous system. The findings of this systematic review were integrated into the paraquat hazard characterization and considered in the point of departure selection and uncertainty factor determination for the Registration Review human health risk assessment (D430827 W. Britton 2019).

In selecting the most sensitive points of departure to estimate risk, the paraquat Registration Review risk assessment accounted for all forms of treatment-related adversity reported for paraquat including the neurotoxic effects discussed in this systematic review. Contact toxicity and adverse effects in the respiratory and renal system reported in OCSPP guideline studies were identified as the most sensitive effects across the toxicity database and relevant paraquat literature. All evidence of nervous system toxicity identified in this systematic review was reported at higher dose levels in animal models. The points of departure selected for risk assessment were established based on the more sensitive respiratory

effects and, therefore, the risk assessment is protective of the neurotoxic effects attributed to paraquat exposure. The findings of this systematic review also do not warrant additional database uncertainty factors for the risk assessment given that there was no evidence to suggest the selected points of departure would be inadequate to protect for the neurotoxic effects.

10.0 Conclusions

As part of the Registration Review process for paraquat, OPP conducted a fit-for-purpose systematic review to evaluate the significance and environmental relevance of the postulated association between paraquat exposure and PD. The literature database that informed the evaluation was compiled from relevant studies identified in the paraquat OPP toxicity database and open literature. Data from the studies were separated into three lines of evidence – human, animal, and *in vitro* – and evaluated for quality, substance, and environmental relevance. Environmental relevance was defined as the likelihood that a given effect would result from an exposure scenario anticipated to occur from typical use of registered paraquat products (e.g. oral including dietary, dermal and inhalation exposure). OPP integrated environmental relevance considerations into the systematic review in order to contextualize hazard information in terms of risk. In total, 26, 11, and 34 relevant studies were considered of sufficient qualtiy to be included in the evaluation of the human, animal, and *in vitro* evidence, respectively, and integrated in the weight of evidence analysis.

Establishing a link between paraquat exposure and PD is reliant on the strength, consistency, and coherence of PD or PD-like hallmarks within and across the human, animal, and *in vitro* lines of evidence, and concordance with toxicokinetic and mechanistic data. Some evidence connecting environmentally relevant paraquat exposure to motor, neuropathological, and/or neurochemical hallmarks of PD was reported in the acceptable literature compiled for this systematic review; however, confidence in these positive findings was diminished by gaps in the dose and temporal concordance, mixed and conflicting results between and across lines of evidence, and unresolved uncertainties in the studies and overall weight of evidence.

The evaluation of 26 epidemiologic articles considered reported findings on 13 study populations, including three agricultural cohorts, nine hospital-based populations, and one PD registry in Nebraska. These study populations may have been exposed to paraquat through occupational and non-occupation exposure pathways that vary in terms of magnitude, frequency, and duration, with occupational study populations being more likely to experience exposure as a result of direct use of paraquat. With respect to occupational exposure, it was determined that there *is limited, but insufficient epidemiologic evidence of a clear associative or causal relationship.* This conclusion was based on mixed findings in both the Agricultural Health Study cohort and other study populations. These studies may all be subject to uncertainty due to limitations in their design, exposure assessment approach, and potential for bias. With respect to non-occupational study populations, evidence from three study populations was evaluated and it was determined that there is *insufficient epidemiologic evidence of a clear associative or causal relationship.* This conclusion was based on the small number of studies on non-occupational populations, lack of consistent evidence of a positive association, and the potential for bias.

Empirical evidence of motor impairment in laboratory animals was observed in male mice following oral exposure for at least 28 days to doses $\geq$7.2 mg ion/kg/day (10 mg dichloride/kg/day). These findings were the strongest evidence of neurotoxicity attributed to paraquat in the animal literature evaluated for this systematic review. The behavioral changes were observed across several studies that used a high purity paraquat product and exhibited a large magnitude of change from controls. Motor impairment was, however, not observed in female mice nor in rats of either sex. Only one animal study presented evidence

to suggest the observed motor impairment in male mice was connected to dopaminergic neuron degeneration and neurochemical disruption – two hallmarks integral to the pathology of PD in humans – and did not provide enough information to evaluate consistency, dose response, or temporal concordance. Toxicokinetic, *in vitro*, and mechanistic data added credibility to the positive findings in male mice but the lack of supporting empirical evidence for tissue, cellular, and biochemical PD-like hallmarks in the animal studies diminish confidence that the observed motor impairment was a result of a PD-like pathology in rodents. Other environmentally relevant routes of exposure were less studied in the literature. No reliable evidence of PD-like hallmarks was observed in mice or rats after repeated intranasal exposure, which was consistent with the toxicokinetic data indicating paraquat did not distribute to the ventral midbrain or striatum after acute exposure. No data were available to evaluate PD-like hallmarks following dermal exposure; however, the systemic paraquat concentration is expected to be low following dermal exposure provided the dermal dose does not achieve levels that affect the integrity of the skin. Overall, the limited, mixed findings in the animal literature were considered weak evidence of a PD-like response to paraquat exposure.

Qualitative similarities in the positive findings for *in vitro* and behavioral outcomes between rodents and humans indicated some interspecies coherence in the neurological response to paraquat exposure; however, there was a lack of coherence for tissue, cellular, subcellular, and biochemical PD hallmarks, in part because few animal studies and no human studies investigated these hallmarks. The small number of positive findings and the lack of consistency in the findings in the human studies also diminished confidence in the biological plausibility of the animal and *in vitro* findings. Occupational and dietary exposure in humans resulting from pesticidal use of paraquat products currently registered in the United States is not estimated to reach external dose levels that elicited PD-like effects in whole animal studies. These estimates may not apply for uses outside of the United States but do suggest that the PD-like outcomes observed in the laboratory are not likely to occur from label-directed pesticidal uses in the US. Given the weakness within and across lines of evidence and the exposure considerations outlined above, OPP concluded that the weight of evidence was insufficient to link paraquat exposure from pesticidal use of US registered products to PD in humans. OPP did not evaluate the adverse outcome pathways (AOP) proposed in the open literature nor develop one from the data gathered in the systematic review. Given the lack of sufficient evidence for a causal association, OPP did not consider an AOP necessary to characterize paraquat toxicity and evaluate risk for registered products.

The findings of this systematic review were integrated with the rest of the paraquat toxicity profile in the hazard characterization and were considered in the point of departure selection and uncertainty factor determination for the Registration Review human health risk assessment. The toxicity profile for paraquat indicates that contact toxicity and effects in the respiratory and renal system occur at lower doses than those eliciting neurotoxicity in animal models. Paraquat is also lethal to pregnant rats at the doses reported to elicit neurotoxicity. Based on these findings, it is expected that a multitude of contact and systemic effects would precede the PD-like neurotoxic effects reported in the literature. Contact, renal, and respiratory toxicity are, therefore, of greater concern to human health and more relevant to assessing risk from paraquat exposure during routine use of pesticidal products with US registration. Points of departure selected for risk assessment were thus based on the more sensitive respiratory effects and are protective of the neurotoxic effects attributed to paraquat exposure discussed in this systematic review.

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Appendix 1 Epidemiology Systematic Review Supplemental Information

A1.1 Search Terms for the OPP Paraquat Epidemiology Review

Table A1.1 Paraquat literature search terms

Paraquat Terms	Health Effects/Disease Terms	Exposure Terms	Methods Terms	Excluded Terms
Paraquat	Health effect*	Expose*	Epidemiolog*	Drosophila
Paraquat[mh]	Health impact*	Environmental exposure[mh]	Epidemiologic methods[mh]	Rat/rats
Methyl viologen	Adverse effects [subheading]	Occupational exposure[mh]	Epidemiologic studies[mh]	Mouse/mice
Gramoxone	Illness*	Prenatal exposure, delayed effects[mh]	Epidemiology [subheading]	Rodent*
	Environmental illness[mh]	Poison*	Case control	Monkey*
	Occupational illness[mh]	Poisoning[subheading]	Retrospective	Zebrafish
	Disease*	Toxic*	Prospective	Trout
	Agricultural workers' diseases[mh]	Intoxication*	Cohort	Fish
	Medical	Toxicity[subheading]	Longitudinal	Foxhound*
	Hospital*	Accident*	Cross-sectional	Bird*
	Mortality	Accidents, occupational[mh]	Incidence[mh]	Sheep
	Death	Inhalation/inhale*	Occupational stud*	Suicid*
	Pregnancy outcome*	Absorb*	Community stud*	Treatment*
	Pregnancy outcome[mh]	Skin absorption[mh]	Environment* stud*	Therap*
	Birth defect*	Contaminat*	Health survey*	Prognostic
	Birth weight	Food contamination[mh]		Prognosis
	Birth weight[mh]	Ingest*		Case report*
	Parkinson/Parkinson's	Consum*/consumption		
	Paralysis agitans	Drink*		
	Parkinson disease[mh]	Water		
	Amyotrophic lateral sclerosis	Herbicides[mh]		
	Neurologi*/neurotoxi*/neurodegenerat*/neuromuscular*	Pesticides[mh]		
	Neurodegenerative disease[mh]			
	Kidney/renal			
	Arthritis			
	Respirat*			
	Pulmonary/lung			
	Thyroid			
	Cardiac /myocardial			
	Cancer*			
	Carcinogen*			
	Neoplasms[mh]			
	Leukemia/myeloma/lymphoma/			
	hodgkin's/sarcoma			
	Cancer sites: prostate/breast/ovar*/			
	colon/colorectal/liver/pancrea*/			
	bladder			

[mh] indicates a Medical Subject Heading(MeSH) in PubMed
[subheading] indicates a qualifier used to describe a specific aspect of a MeSH heading
* indicates truncation (i.e., that alternate endings were searched)

A1.2 Epidemiology Literature Screening Inclusion/Exclusion Criteria

Table A1.2 Inclusion/Exclusion Criteria for OPP Paraquat Epidemiology Review

Inclusion Criteria	Exclusion Criteria (or blank if none)
Participants/Population	
– Humans with no restrictions, including no restrictions on age, life stage, sex, country of residence/origin, race/ethnicity, lifestyle, or occupation	– Studies reporting outcomes for non-human study subjects – Experimental model or *in vitro* studies – Fate and transport studies
Exposure	
– Exposure studied must be paraquat in any application via any route of exposure	– No paraquat-specific investigation (e.g. general herbicide only)
Comparators	
– Exposed or case populations must be compared to a population with low/no exposure or to non-cases to arrive at a risk/effect size estimate of a health outcome associated with paraquat exposure	
Outcomes	
– All reported human health effects, with no restrictions on human system affected (effects could be based on survey or other self-report, medical records, biomarkers, publicly available health data, or measurements from human sample populations	– Reported outcomes other than human health effects (e.g., environmental measures)[1] – Acute poisonings and overexposure studies – No risk/effect estimate reported
Publications (e.g., language restrictions, use of conference abstracts, etc.)	
– Report must contain original data – Abstract is written in the English language	– Articles with no original data (e.g., editorial or review[2]) – Studies published in abstract form only (grant awards, conference abstracts) – Studies not peer- reviewed – Retracted articles

[1] For the purposes of the epidemiology literature review, the agency considered human health effects via the toxicological paradigm presented by the NRC as pathologies or health impairments subsequent to altered structure/function (Henderson et al. 1987). Thus, studies with outcomes of altered structure (e.g., DNA alteration, sister chromatid exchange, cell proliferation) or biomarker or other exposure outcomes (e.g., in breast milk, urine, cord blood, or plasma) that did not also include an associated health pathology (e.g., cancer, asthma, birthweight) failed to meet the inclusion criteria for "human health effects" for the purposes of this epidemiology literature review.

[2] Relevant reviews are used as background and for reference scanning.

A1.3 Epidemiology Study Evaluation Criteria

Table A1.3: Epidemiology Study Quality Considerations.

Parameter	High	Moderate	Low
Exposure assessment	Exposure assessment includes information on paraquat or metabolite in the body, quantitative air sample data, or high quality questionnaire on chemical-specific exposure assessment during relevant exposure window	Questionnaire based individual level information on paraquat	Low quality questionnaire-based exposure assessment, or ecologic exposure assessment, with or without validation
Outcome Assessment	Standardized tool, validated in study population; or, medical record review with trained staff	Standardized tool, not validated in population, or screening tool; or, medical record review, methods unstated	Subject report, without additional validation
Confounder control	Good control for important confounders relevant to paraquat study question, and standard confounders	Moderately good control of confounders, standard variables, not all variables for paraquat study question	Multi-variable analysis not performed, no adjustments
Statistical Analysis	Appropriate to study question and design, supported by adequate sample size, maximizing use of data, reported well (not selective)	Acceptable methods, questionable study power (esp. sub-analyses), analytic choices that lose information, not reported clearly	Minimal attention to statistical analyses, comparisons not performed or described clearly
Risk of (other) bias (selection, differential misclassification, other)	Major sources of other potential biases not likely present, present but analyzed, unlikely to influence magnitude and direction of the risk estimate	Other sources of bias present, acknowledged but not addressed in study, may influence magnitude but not direction of estimate	Major study biases present, unacknowledged or unaddressed in study, cannot exclude other explanations for study finding

Note: Overall study quality ranking based on comprehensive assessment across the parameters.

Appendix 2 NTP Scoping Review Supplemental Information

A2.1 Search Terms for NTP Scoping Review

Search terms used in the NTP Scoping Review were reproduced from the NTP Scoping Review protocol (NTP 2018) with permission from the authors. The search terms, the date of the search, and the number of citations returned for each electronic database searched are provided below.

EMBASE

Date of search: March 29, 2017; 107 results

(paraquat OR 1910-42-5 OR gramoxone OR methyl-viologen OR paragreen-A)

AND

(alpha-synuclein OR apoptosis OR astrocyte OR astrocytes OR ataxia OR autophagy OR axon OR axonal OR axons OR bradykinesia OR brain OR central-nervous OR dendrite OR dendrites OR dentritic OR dj-1 OR dopamine OR dopaminergic OR gait OR ganglia OR glial OR gliosis OR glutamate OR glutamates OR Glutamic Acids OR glutathione OR Lewy bodies OR lewy body OR locomotion OR locomotor-activity OR lrrk2 OR Mesencephalon OR Mesencephalons OR microglia OR microglial OR microglials OR midbrain OR mitochondria OR Mitochondrial OR Mitochondrion OR motor-activity OR mpp OR mptp OR NADPH-oxidase OR nerve OR nerves OR nervous OR neural OR neurobehavior OR neurobehavioral OR neurobehaviour OR neurobehavioural OR neuroblastoma OR neurodegeneration OR neurodegenerative OR neuroglia OR neurological OR neuromotor OR neuron OR neuronal OR neuronopathy OR neurons OR neuropathies OR neuropathology OR neuropathy OR neurotoxic OR neurotoxicity OR neurotransmitter OR neurotransmitters OR nigral OR nigrostriatal OR nitric-oxide OR nitrosative-stress OR oxidative-stress OR paralysis-agitans OR parkin OR parkinson OR parkinsons OR parkinsonian OR parkinsonism OR pink1 OR reactive-oxygen-species OR rigidity OR snpc OR striatal OR striatum OR substantia-nigra OR synapse OR synapses OR synaptic OR synuclein OR synucleins OR tau OR tauopathies OR tauopathology OR tauopathy OR Thioredoxin-Disulfide OR thioredoxin-reductase OR tremor OR tremors OR Tyrosine 3-Monooxygenase OR tyrosine-hydroxylase OR ubiquitin)

PubMed

Date of search: March 29, 2017; 3,501 results

(paraquat[tiab] OR paraquat[mh] OR gramoxone[tiab] OR methyl-viologen[tiab] OR paragreen-A[tiab])

AND

(alpha-synuclein[tiab] OR alpha-synuclein[mh] OR apoptosis[tiab] OR apoptosis[mh] OR astrocyte[tiab] OR astrocytes[tiab] OR astrocytes[mh] OR ataxia[tiab] OR autophagy[tiab] OR autophagy[mh] OR axon[tiab] OR axonal[tiab] OR axons[tiab] OR axons[mh] OR bradykinesia[tiab]

OR brain[tiab] OR central-nervous[tiab] OR dendrite[tiab] OR dendrites[tiab] OR dentritic[tiab] OR dj-1[tiab] OR dopamine[mh] OR dopamine[tiab] OR Dopamine Plasma Membrane Transport Proteins[mh] OR dopaminergic[tiab] OR gait[tiab] OR gait[mh] OR ganglia[tiab] OR glial[tiab] OR gliosis[tiab] OR gliosis[mh] OR glutamate[tiab] OR glutamates[mh] OR glutamates[tiab] OR Glutamic Acids[tiab] OR glutathione[tiab] OR glutathione[mh] OR Lewy bodies[tiab] OR lewy body[tiab] OR locomotion[mh] OR locomotion[tiab] OR locomotor-activity[tiab] OR lrrk2[tiab] OR Mesencephalon[tiab] OR Mesencephalons[tiab] OR microglia[tiab] OR microglial[tiab] OR microglials[tiab] OR midbrain[tiab] OR mitochondria[tiab] OR mitochondria[mh] OR Mitochondrial[tiab] OR Mitochondrion[tiab] OR motor-activity[tiab] OR motor-activity[mh] OR mpp[tiab] OR mptp[tiab] OR NADPH-oxidase[mh] OR NADPH-oxidase[tiab] OR nerve[tiab] OR nerves[tiab] OR nervous[tiab] OR nervous-system[mh] OR nervous-system-diseases[mh] OR nervous-system-physiological-processes[mh] OR neural[tiab] OR neurobehavior[tiab] OR neurobehavioral[tiab] OR neurobehaviour[tiab] OR neurobehavioural[tiab] OR neuroblastoma[tiab] OR neuroblastoma[mh] OR neurodegeneration[tiab] OR neurodegenerative[tiab] OR neuroglia[tiab] OR neurological[tiab] OR neuromotor[tiab] OR neuron[tiab] OR neuronal[tiab] OR neuronopathy[tiab] OR neurons[tiab] OR neuropathies[tiab] OR neuropathology[tiab] OR neuropathy[tiab] OR neurotoxic[tiab] OR neurotoxicity[tiab] OR neurotransmitter[tiab] OR neurotransmitter agents[mh] OR neurotransmitter agents[Pharmacological Action] OR neurotransmitters[tiab] OR nigral[tiab] OR nigrostriatal[tiab] OR nitric-oxide[tiab] OR nitric-oxide[mh] OR nitric-oxide-synthase[mh] OR nitrosative-stress[tiab] OR oxidative-stress[tiab] OR paralysis-agitans[tiab] OR parkin[tiab] OR parkin protein[supplementary concept] OR parkinson[tiab] OR parkinsons[tiab] OR parkinson's[tiab] OR parkinsonian[tiab] OR parkinsonism[tiab] OR pink1[tiab] OR reactive-oxygen-species[tiab] OR reactive-oxygen-species[mh] OR rigidity[tiab] OR snpc[tiab] OR striatal[tiab] OR striatum[tiab] OR substantia-nigra[tiab] OR synapse[tiab] OR synapses[tiab] OR synaptic[tiab] OR synuclein[tiab] OR synucleins[tiab] OR synucleins[mh] OR tau[tiab] OR tau proteins[mh] OR tauopathies[tiab] OR tauopathology[tiab] OR tauopathy[tiab] OR Thioredoxin-Disulfide[tiab] OR Thioredoxin-Disulfide Reductase[mh] OR thioredoxin-reductase[tiab] OR tremor[tiab] OR tremors[tiab] OR Tyrosine 3-Monooxygenase[mh] OR Tyrosine 3-Monooxygenase[tiab] OR tyrosine-hydroxylase[tiab] OR ubiquitin[tiab] OR ubiquitin[mh])

Web of Science

Date of search: March 29, 2017; 3,551 results

All terms searched in Title, Abstract, or Keywords

LIMITS:

Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, CCR-EXPANDED, IC

Timespan=All years

(paraquat OR 1,1'-Dimethyl-4,4'-bipyridinium-dichloride OR 1910-42-5 OR gramoxone OR methyl-viologen OR paragreen-A)

AND

(alpha-synuclein OR apoptosis OR astrocyte OR astrocytes OR ataxia OR autophagy OR axon OR axonal OR axons OR bradykinesia OR brain OR central-nervous OR dendrite OR dendrites OR dentritic OR dj-1 OR dopamine OR dopaminergic OR gait OR ganglia OR glial OR gliosis OR glutamate OR glutamates OR Glutamic Acids OR glutathione OR Lewy bodies OR lewy body OR locomotion OR locomotor-activity OR lrrk2 OR Mesencephalon OR Mesencephalons OR microglia OR microglial OR microglials OR midbrain OR mitochondria OR Mitochondrial OR Mitochondrion OR motor-activity OR mpp OR mptp OR NADPH-oxidase OR nerve OR nerves OR nervous OR neural OR neurobehavior OR neurobehavioral OR neurobehaviour OR neurobehavioural OR neuroblastoma OR neurodegeneration OR neurodegenerative OR neuroglia OR neurological OR neuromotor OR neuron OR neuronal OR neuronopathy OR neurons OR neuropathies OR neuropathology OR neuropathy OR neurotoxic OR neurotoxicity OR neurotransmitter OR neurotransmitters OR nigral OR nigrostriatal OR nitric-oxide OR nitrosative-stress OR oxidative-stress OR paralysis-agitans OR parkin OR parkinson OR parkinsons OR parkinson's OR parkinsonian OR parkinsonism OR pink1 OR reactive-oxygen-species OR rigidity OR snpc OR striatal OR striatum OR substantia-nigra OR synapse OR synapses OR synaptic OR synuclein OR synucleins OR tau OR tauopathies OR tauopathology OR tauopathy OR Thioredoxin-Disulfide OR thioredoxin-reductase OR tremor OR tremors OR Tyrosine 3-Monooxygenase OR tyrosine-hydroxylase OR ubiquitin)

SCOPUS

Date of search: March 29, 2017; 128 results

All terms searched in Title, Abstract, or Keywords

LIMITS:

Indexes=SCI-EXPANDED, SSCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, CCR-EXPANDED, IC

Timespan=All years

(paraquat OR 1,1'-Dimethyl-4,4'-bipyridinium-dichloride OR 1910-42-5 OR gramoxone OR methyl-viologen OR paragreen-A)

AND

(alpha-synuclein OR apoptosis OR astrocyte OR astrocytes OR ataxia OR autophagy OR axon OR axonal OR axons OR bradykinesia OR brain OR central-nervous OR dendrite OR dendrites OR dentritic OR dj-1 OR dopamine OR dopaminergic OR gait OR ganglia OR glial OR gliosis OR glutamate OR glutamates OR Glutamic Acids OR glutathione OR Lewy bodies OR lewy body OR locomotion OR locomotor-activity OR lrrk2 OR Mesencephalon OR Mesencephalons OR microglia OR microglial OR microglials OR midbrain OR mitochondria OR Mitochondrial OR Mitochondrion OR motor-activity OR mpp OR mptp OR NADPH-oxidase OR nerve OR nerves OR nervous OR neural OR neurobehavior OR neurobehavioral OR neurobehaviour OR neurobehavioural OR neuroblastoma OR neurodegeneration OR neurodegenerative OR neuroglia OR neurological OR

neuromotor OR neuron OR neuronal OR neuronopathy OR neurons OR neuropathies OR neuropathology OR neuropathy OR neurotoxic OR neurotoxicity OR neurotransmitter OR neurotransmitters OR nigral OR nigrostriatal OR nitric-oxide OR nitrosative-stress OR oxidative-stress OR paralysis-agitans OR parkin OR parkinson OR parkinsons OR parkinson's OR parkinsonian OR parkinsonism OR pink1 OR reactive-oxygen-species OR rigidity OR snpc OR striatal OR striatum OR substantia-nigra OR synapse OR synapses OR synaptic OR synuclein OR synucleins OR tau OR tauopathies OR tauopathology OR tauopathy OR Thioredoxin-Disulfide OR thioredoxin-reductase OR tremor OR tremors OR Tyrosine 3-Monooxygenase OR tyrosine-hydroxylase OR ubiquitin)

Toxline

Date of search: March 29, 2017; 1,089 results

All terms searched in Title, Abstract, or Keywords

LIMITS:

Exclude PubMed Records

Do NOT add chemical synonyms and CASRNs to search

Search exact words

(paraquat OR 1,1'-Dimethyl-4,4'-bipyridinium-dichloride OR 1910-42-5 OR gramoxone OR methyl-viologen OR paragreen-A)

AND

(alpha-synuclein OR apoptosis OR astrocyte OR astrocytes OR ataxia OR autophagy OR axon OR axonal OR axons OR bradykinesia OR brain OR central-nervous OR dendrite OR dendrites OR dentritic OR dj-1 OR dopamine OR dopaminergic OR gait OR ganglia OR glial OR gliosis OR glutamate OR glutamates OR Glutamic Acids OR glutathione OR Lewy bodies OR lewy body OR locomotion OR locomotor-activity OR lrrk2 OR Mesencephalon OR Mesencephalons OR microglia OR microglial OR microglials OR midbrain OR mitochondria OR Mitochondrial OR Mitochondrion OR motor-activity OR mpp OR mptp OR NADPH-oxidase OR nerve OR nerves OR nervous OR neural OR neurobehavior OR neurobehavioral OR neurobehaviour OR neurobehavioural OR neuroblastoma OR neurodegeneration OR neurodegenerative OR neuroglia OR neurological OR neuromotor OR neuron OR neuronal OR neuronopathy OR neurons OR neuropathies OR neuropathology OR neuropathy OR neurotoxic OR neurotoxicity OR neurotransmitter OR neurotransmitters OR nigral OR nigrostriatal OR nitric-oxide OR nitrosative-stress OR oxidative-stress OR paralysis-agitans OR parkin OR parkinson OR parkinsons OR parkinson's OR parkinsonian OR parkinsonism OR pink1 OR reactive-oxygen-species OR rigidity OR snpc OR striatal OR striatum OR substantia-nigra OR synapse OR synapses OR synaptic OR synuclein OR synucleins OR tau OR tauopathies OR tauopathology OR tauopathy OR Thioredoxin-Disulfide OR thioredoxin-reductase OR tremor OR tremors OR Tyrosine 3-Monooxygenase OR tyrosine-hydroxylase OR ubiquitin)

A2.2 NTP Scoping Review Screening Inclusion/Exclusion Criteria

The inclusion/exclusion criteria used in the NTP Scoping Review were reproduced from the NTP Scoping Review protocol (NTP 2018) with permission from the authors.

Table A2.2 Inclusion/Exclusion Criteria NTP Scoping Review

Evidence Stream	Inclusion Criteria	Exclusion Criteria (or blank if none)
Participants/Population (Human Studies or Experimental Model Systems)		
Human	− No restrictions on sex, age, life stage (including in utero exposure) at time of exposure or outcome assessment − No restrictions on country of residence/origin, lifestyle, race/ethnicity, or occupation	
Animal	− No restrictions on sex, age, species (including Drosophila), or life stage at exposure or outcome assessment	− Studies in non-animal organisms (e.g., plants, fungi, protists, bacteria)
In vitro	− Studies involving an *in vitro* exposure system and neurological measures directed at cellular, biochemical, and molecular mechanisms that may explain how exposure to paraquat leads to Parkinson's disease	
Exposure		
Human	− Exposure to paraquat dichloride (CAS# 1910-42-5) based on administered dose or concentration, biomonitoring data (e.g., urine, blood, or other specimens), environmental measures (e.g., air, water levels), or indirect measures (e.g., job title)	
Animal	− Exposure to paraquat dichloride (CAS# 1910-42-5) based on administered dose or concentration or bio-monitoring data (e.g., urine, blood, or other specimens) − No restrictions on route of administration	
In vitro	− Exposure to paraquat dichloride based on administered dose or concentration	
Comparators		
Human	− Humans exposed to lower levels (or no exposure/exposure below detection levels) of paraquat dichloride	
Animal	− Study must include vehicle or untreated control group	
In vitro	− Study must include vehicle or untreated control group	
Outcomes		
Human	**Primary outcomes** ***[following in vivo exposure to paraquat dichloride]:*** − Diagnosis of Parkinson's disease and/or clinical observations, neurobehavioral, or neuropathological outcomes typically associated with Parkinson's disease or parkinsonism following *in vivo* exposure, focusing on tissue level and functional abnormalities, descriptive and/or functional assessment of the central nervous system, including the nigrostriatal (dopamine) system. Examples of relevant outcomes include tremor, bradykinesia, rigidity, postural instability, and any other movement abnormalities associated with parkinsonism.	− Studies reporting on toxicity in organs or tissues not associated with the central or peripheral nervous system
	Secondary outcomes ***[following in vivo exposure to paraquat dichloride]:***	

Table A2.2 Inclusion/Exclusion Criteria NTP Scoping Review

Evidence Stream	Inclusion Criteria	Exclusion Criteria (or blank if none)
	– Tissue, cellular, biochemical, and/or molecular outcomes resulting from *in vivo* exposure that have a mechanistic association with Parkinson's disease or are evidence of toxicity in the nervous system but are not specific to Parkinson's disease.	
Animal	**Primary outcomes** ***[following in vivo exposure to paraquat dichloride]:*** – Neurobehavioral or neuropathological outcomes, focusing on whole body and tissue level abnormalities typically associated with Parkinson's disease following *in vivo* exposure. Endpoints include motor activity and coordination, sensorimotor reflexes, effects on cognitive function, quantitative or qualitative assessment of dopaminergic neuron counts in the substantia nigra and dopaminergic neuron terminals in the striatum, and other descriptive and/or functional assessments of the central nervous system including the nigrostriatal (dopamine) system that are considered hallmarks of Parkinson's disease (e.g., detection of intracytoplasmic Lewy bodies).	
	Secondary outcomes ***[following in vivo exposure to paraquat dichloride]:*** – Tissue, cellular, biochemical, and/or molecular outcomes resulting from *in vivo* exposure that have a mechanistic association with Parkinson's disease (e.g. dopamine and metabolite levels in the nigrostriatal pathway, TH+ immunoreactivity density) or are evidence of toxicity in the nervous system, but are not specific to Parkinson's disease (e.g. oxidative stress, inflammation, mitochondrial and/or proteasomal dysfunction).	
In vitro	***Following in vitro exposure to paraquat dichloride:*** – *In vitro* assays investigating either cellular or molecular responses commonly attributed to Parkinson's disease (e.g. assessment of functionality, integrity, and viability for nerve cells critical to the nigrostriatal (dopamine) system) or generic cellular responses commonly attributed to paraquat exposure but are not unique to Parkinson's disease (e.g. measures of oxidative stress and mitochondria dysfunction in nerve cells, epigenetic changes). – Mechanistic assays investigating proposed pathways for the etiology of Parkinson's disease (e.g. enzyme interactions, cell signaling)	– Studies reporting on toxicity unrelated to the central or peripheral nervous system
Publications (e.g., language restrictions, use of conference abstracts, etc.)		
Human, Animal or *In vitro*	– Report must contain original data – Studies published in a language other than English will be collected and categorized by health effect or mechanism to the extent they can be categorized without full translation as extensive translation and level of effort are beyond the goals of this scoping review.	– Articles with no original data (e.g., editorial or review[1]) – Studies published in abstract form only (grant awards, conference abstracts) – Retracted articles – Non-English language articles that cannot be

Table A2.2 Inclusion/Exclusion Criteria NTP Scoping Review

Evidence Stream	Inclusion Criteria	Exclusion Criteria (or blank if none)
		categorized based on English abstract

[1]Relevant reviews are used as background and for reference scanning.

Appendix 3 Communication with NTP Experts

OPP communicated with Drs. Robert Sills, and Jau-Shyong Hong from NTP and Dr. Peter Little, a neuropathologist contracted with NTP, to address questions relating to study methodology and PD pathology. The purpose of this communication was to gain clarification on several topics that were critical to decisions made during the open literature screen, review of study quality, and analyzing the weight of evidence. OPP reached out to these scientists in particular because of their expertise in the fields of neuropathology and PD. OPP communicated with the experts twice by phone over the course of the systematic review process (on March 29, 2018 and June 13, 2018) and a summary of those communications is presented below. It should be noted that the experts did not have direct input on the open literature screen, study quality review, or weight of evidence analysis. The conclusions presented in this systematic review memo are those of OPP alone and do not reflect the opinion of the experts nor NTP.

A3.1 Summary of the Communication with NTP Experts

OPP asked a series of questions during the conference calls relating to general neuropathology and PD. These questions are bolded below and are followed by the response from the experts in italics.

1. **The animal portion of the systematic review will focus on three endpoints to examine the link between PQ exposure and PD: dopaminergic neuron and terminal degeneration, changes in neurotransmitter levels in the striatum, and motor activity changes. There are several other effects that are examined in the ~100 primary animal literature studies that might be considered hallmarks of PD; however, the selected endpoints are assessed most frequently. Are these three endpoints sufficient to describe a Parkinson's-like response to exposure in laboratory animal studies or are there other hallmarks of PD that are needed to build a stronger weight of evidence? We will also discuss the *in vitro* and mechanistic data in the context of hypothesized modes of action but will not be developing our own adverse outcome pathway or mode of action argument in this review.**

Dopaminergic neuron (DA neuron) and terminal degeneration, changes in neurotransmitter levels in the striatum, and motor activity changes are the primary endpoints for Parkinson's disease (PD). Pathological verification of PD in humans also includes identification of Lewy bodies or alpha synuclein aggregation; however, the rodent literature on this outcome is not as clear as it is for humans. Nevertheless, studies that report alpha synuclein aggregation/Lewy body data will be included in the discussion of animal primary outcomes. In addition, focusing on the nigrostriatal pathway may be too limiting and the weight of evidence should consider neurodegenerative responses in related areas of the brain including the olfactory pathway or other non-motor syndromes.

2. **We have also extracted data for non-motor activity behavioral changes (i.e. anxiety, olfactory changes, cognitive change). There are fewer studies with this information, so it won't be the focus of our discussion; however, given that some of these changes are observed in patients during the early stages of PD, please provide some suggestions for how EPA should evaluate and weigh these endpoints. Additionally, there are a few studies that evaluate cognitive function, but use measures that could be affected by motor function (e.g., escape latency in the Morris Water Maze). Please provide some suggestions for how EPA should evaluate and weigh these endpoints.**

Non-motor activity responses including olfactory dysfunction, depression, and anxiety are observed in humans with clinically diagnosed PD and are a more recent focal point of PD research. Given its importance in the manifestation of human PD, non-motor activity behavior outcomes should be included as part of the weight of evidence. In addition, motor activity should be evaluated concurrently with cognitive function in behavioral assessments that rely in some part on animal movement (e.g. Morris Water Maze). Otherwise, it will be difficult to discern the true impact of PQ exposure on cognitive function.

3. **Our systematic review discussion will focus primarily on the nigrostriatal pathway. A number of studies also present information on other brain regions. Do you have any suggestions for how EPA should consider vulnerabilities, changes, or effects in other regions of the brain with respect to development and progression of PD?**

Evidence of neuronal degeneration in other areas of the brain is important in the developmental of PD in humans and is well established in the literature. Studies using PD laboratory animal models have demonstrated increased vulnerability of norepinephrine neurons projecting from the locus coeruleus (LC) compared to DA neurons in the substantia nigra. Similar effects have been reported in the LC for the PQ PD model (Fernagut et al. 2007). Recommend including discussion of studies that report neuronal degeneration in other areas of the brain in the weight of evidence discussion for the systematic review.

4. **Most studies used stereology to estimate the number of neurons in the SNpc; however, there were several studies used density of the tyrosine hydroxylase immunoreactivity as a surrogate measure of neuron count (Ren et al. 2009). Similarly, damage to striatal terminals was assessed using optical density and fiber counts (Fernagut et al. 2007 used both measures). Although we will report the results of both measures, we intend to focus our discussion on the stereology and fiber count data because we thought they were more reliable. Is that a reasonable approach? How should we consider the two methods of estimating neurons/terminal counts?**

Stereology is the most robust measure of neuronal cell loss, but the optical density measurements should not be discounted and provide additional weight to the discussion, especially when the data are consistent with the stereology data. Fiber counts and OD measurements of terminals in the striatum should be treated similarly. Neurotransmitter levels are often more important to the development of PD than neuron cell counts. In addition to neurotransmitter levels, the TH protein content in the striatum and SN should be examined because this enzyme is the rate limiting step in dopamine formation and would provide information on the limits of dopamine synthesis in the SN and striatum.

5. **There are several industry published studies in our primary literature review (Breckinridge et al. 2013 and Minnema et al. 2014) that were also submitted to the agency prior to publication. These two studies assess neuron degeneration in the common mouse model using stereology for neuron counts and stains for neuropathology assessment. Interestingly, they mostly present null results using an exposure design similar to studies in the literature that report significant decline in dopaminergic neuron counts in the SNpc. We are interested in figuring out why there are differences between the results in these studies and others in the literature. To that end we have a number of questions about stereology techniques and other methodology choices that**

could impact the results. Many of these are identified by Smeyne et al. (2016) – another industry funded study that reported null results in the mouse model – as potential confounding factors that could account for the differences in reported results across the literature (see also the Smeyne et al. (2016) supplemental documents which includes discussion of a systematic review conducted by Smeyne et al. (2016) that examined published literature on Parkinson's research using the same PQ mouse model).

a. **General Toxicity**
    i. **Are there any specific types of animal confounders (i.e. sex of the rat, food/water consumption, etc.) that might impact the behavioral/neurological responses we selected as our endpoints of interest?**

The three studies identified above were all conducted by the same group and sponsored by Syngenta. The neuropathology was conducted by board certified pathologists and the studies were peer reviewed by well-known pathologists. Males are often more sensitive compared to females. A majority of the PQ PD literature is conducted on male laboratory animals; thus, this confounding factor is not anticipated to impact the overall body of literature. Do not know of other confounding factors related to PD or PQ exposure.

b. **Neuropathology**
    i. **Are the stains used in the Breckinridge et al. (2013) and Minnema et al. (2014) commonly used for neuropathology and are they reliable? We note that the Breckinridge et al. (2013) study observed significant decrease in neurons after 3 doses of 15 mg/kg (3x15) exposure but there was no evidence of neuron degeneration in the neuropathology examination. This is further complicated by the lack of neuron loss observed after 3 doses of 25 mg/kg. The latter inconsistency might speak more to a lack of confidence in the neuron loss observation at 3x15**

The neuropathology stains employed in the Breckinridge et al. (2013) and Minnema et al. (2014) studies are commonly used and reliable. The application of the technology in these papers is fine and they should be commended on their comprehensive evaluation of cell types and use of up-to-date cell markers.

    ii. **Would we expect an increase in microglia and/or astrocyte reactivity concurrent with dopaminergic neuron cell loss? This may end up being more of a study interpretation question as to whether this would be a confirmatory measure or more mechanistic information.**

Microglia and astrocyte reactivity is considered a quality indicator of neuroinflammation. Microglia responds first to damage in the nervous system, usually within 24-48 hours, and persists. Astrocytes can be seen within a few days of the damage and the reactivity increases in intensity over 3 weeks. Both are important signaling for neurodegeneration processes. The timing of the assay for microglia and astrocyte reactivity is important and it is more constructive to assess the activity of both markers. The timing of the microglia and/or astrocyte assessment should be scrutinized, particularly when only one of the markers is examined. The length and intensity of the microglial or astrocyte response will depend on the duration of the exposure and the dose.

c. **Stereology**

i. **Is there a preferred tag for stereology? We've noted that both chromogen and fluorescent tags are used and can produce inconsistent results as was the case in the Breckenridge et al. (2013) study.**

NTP reached out to Danielle Brown from Charles River to comment on the stereology questions as she has more experience with the techniques and procedures. She commented that the brightfield DAB stain technique is preferred over fluorescence because the tissue anatomy can be viewed better and the regions of interested more easily delineated. Her lab tends to use virtual slides (whole scanned slides) and physical dissectors with the Visiophaarm system (rather than thick optical dissectors as done in the Breckenridge paper). They also use Proportionator (image analysis algorithm guided sampling) to increase efficiency and statistical power.

ii. **Is one stereology method more reliable than the other (3-D versus 2-D as described in Smeyne et al. (2016)?**

Danielle highly discouraged the model based (2-D) stereology methods used in the Smeyne et al. 2016 study. Without the dissector method there is always bias in counting objects according to the shape and orientation. Consequently, the model-based method is less reliable.

iii. **Smeyne et al. (2016) looked at a number of stereology parameters including thickness of sections, number of sections sampled, dissector height, and guard zone (Supplemental to Smeyne et al. 2016 publication). Although this may result in inter-study variability in the neuron counts, could we assume that intra-animal count variability within a study would be low if these parameters are held constant throughout the stereology analysis?**

Danielle mentioned that her lab often has difficulty consistently and accurately delineating between two adjacent areas with the same antigenic profile in the brain. She uses an atlas to differentiate, but it is not perfect and still subject to human error. This may contribute to inconsistent results observed within a study (such as in the Breckinridge et al. 2013 study) and across studies. Methods (e.g. counterstains) are currently in development to help resolve this issue and improve accuracy of stereology studies in the brain.

iv. **In your experience, is it difficult to detect the Nissl strain cells if you are also tagging TH+ neurons? Is this only a problem with chromogen or can this this be circumvented with fluorescent tags?**

The Nissl and chromogen tag techniques are compatible. Therefore, researchers should be able to differentiate between the two for determining TH+ neurons and total neuron counts and this should not be considered a confounding factor.

v. **A number of studies mention using the optical fractionator method for stereology counting and claim it is an unbiased method of counting. However, many of these studies also do not blind the pathologist to treatment group. Please comment on the extent, if any, to which the optical fractionator method affects the detection bias introduced by not blinding the researcher counting the neurons.**

The optical fractionator is an unbiased method of counting that is used by Danielle's lab. It focuses down through thick sections on high magnification oil objective and count cells when they come into crisp focus. Danielle's lab also used physical dissectors, which counts cells using matching high magnification fields of consecutive thin sections, and only count cells if it is in one field and not the other. Both methods are unbiased. However, neither eliminate the detection bias introduced by the lack of blinding to treatment group. Danielle strongly recommends blinding the researcher conducting the stereology.

vi. Can you recommend a literature study(ies) that, in your expert opinion, describe sound stereology methods and can be used as a standard to assess the quality of the methods in the studies from our systematic review?

The information provided by Danielle B. from Charles River and NTP should be adequate to assess the quality of stereology methods in the primary outcome literature.

6. As part of our study quality assessment, we need to know if the sample size selected is adequate to measure the magnitude of change that would lead to the manifestation of PD. Please comment on the magnitude (% change, if possible since that is how much of our data are presented) for DA neuron degeneration in the SN and DA levels decreases in the striatum that is likely or known to elicit PD.

Studies of neurodegeneration in human PD cases, historically, have reported at least 50% loss of SN neurons and at least 80% decline in DA levels in the striatum. This magnitude of neuron loss and DA level decline is generally not replicated in the animal models including the MPP+ model. PQ is not well absorbed and only a small fraction accumulates in the brain following exposure (few tenths of a %). Therefore, it is unlikely that the magnitude of SN neuron loss and DA level decline observed in humans can be replicated at sublethal doses of PQ given its toxicity profile. However, more recent studies have shown that the neuron loss reported in human PD cases may have been overestimated and is closer to 30-35%. Recent findings indicate that downregulation of TH also occurs in the SN of PD patients and it is possible that previous cell counts mistook this down regulation for neuron degeneration. It has also been shown that animals can recover from exposure to MPP+ if left alone for some time after exposure ends. The more recent reports of neuron loss (30-35%) are closer to the average loss of TH+ neurons from PQ exposure (~25%; generally, from IP administration) reported in the PQ PD literature.

In addition to answering our questions, the experts provided the following comments on several studies discussed at the meetings.

Ren et al. (2009) *is a well examined study of MPTP and PQ (10mg/kg daily) vs controls in a 4-month mouse oral exposure experiment. The real value of this study is the subchronic exposure rather than acute used in the following 3 studies by Syngenta the maker of paraquat. The data is compelling showing Parkinsonian-like effects on neurobehavior as well as immunohistochemical neuronal evidence and neurochemical evidence of negative effects on striatal neurons. In all cases the reduction of neurons and neurochemical parameters indicates a ~50% reduction in both MPTP and PQ from controls.*

Breckinridge et al. (2013) *is a time limited examination of the effects of MPTP and PQ delivered by weekly IP injections to mice for 1, 2 or 3 weeks at doses of 10,15 or 25 mg/kg/week. The shortcoming of this study that suggested that PQ had no effect on striatal neuronal numbers etc compared to MPTP is that the study design is too short and dosing too infrequent to demonstrate potential neural effects of PQ. The methodology and neuropathology is well conducted but expectedly shows no effects. The fact that the paper is conducted by industry personnel also detracts from the credibility of the study.*

Minnema et al. (2014) *is a subchronic oral exposure to male and female mice daily for 13 weeks at doses of 10 and 30 mg/Kg. This experimental protocol is an improvement over the Breckenridge paper done a year earlier by the similar industrial personnel. The dose and number of IP treatments of MPTP was acute 7 days prior to termination of the study. Four IP injections were given 2 hours apart at 10mg/kg. The methodology of tissue examination is well conducted and the results are at odds with the Ren et al. 2009 article indicating no effect on striatal neurons or associated neurochemical evaluations. The fact that the paper is conducted by the same industry personnel detracts from the credibility of the study.*

In the ***Smeyne et al. (2016)*** *study, PQ was administered by intraperitoneal injections; either once (20 mg/kg) or twice (10 mg/kg) weekly for 3 weeks in two inbred strains of 9- or 16-week old male C57BL/6 mice. Six of the 12 authors are Syngenta employees. This study is well conducted and attempts to vary some conditions of the study which were blinded to the investigators. The results are compelling and under the dosing conditions used show a distinct effect of MPTP but not paraquat on DA neurons. The fact that the paper is conducted by the same industry personnel detracts from the credibility of the study. The experts' opinion is that while this study contradicts other studies that do show a paraquat effect at comparable doses that this and other studies do not examine long term effects of paraquat on the striatum and SN pars compacta DA neurons. Additional studies that look at paraquat effects on brain DA neurons using low mid and high doses over subchronic 90 day and chronic 2-year studies are in order since that would better replicate natural human exposure. The limits of life span of rodents compared to man is a complicating minimizing factor in the role of many neurodegenerative agents that man is exposed to over lifetime.*

The use of MPTP for comparison with paraquat is very questionable since that is like comparing cyanide gas effects to that of methane. There is a real need to compare agents of similar known neurotoxicity over long term experiments